ฤทธิ์ระงับปวดของสิ่งสกัดด้วยเอธานอลจากใบมะม่วงหิมพานต์

นางสาวจันทร์จุรีย์ เขาพินพฤกษ์

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ANALGESIC EFFECTS OF THE ETHANOLIC EXTRACT FROM *ANACARDIUM OCCIDENTALE L*. LEAVES

MISS JANJUREE KAOPINPRUCK

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ได้มีการนำสิ่งสกัดจากใบมะม่วงหิมพานต์มาใช้เป็นยาสมุนไพรพื้นบ้าน ในการรักษาอาการเจ็บเหงือก และอาการปวดฟัน ในการทดลองครั้งนี้มุ่งศึกษาฤทธิ์ระงับปวดของสิ่งสกัดจากใบมะม่วงหิมพานต์ขนาดต่างๆกันในหนูถีบจักร โดยทดลองวางหนูถีบจักรบนแผ่นร้อนและจับเวลาที่หนูถีบจักรสามารถทนอยู่บนแผ่นร้อนได้ (hot-plate test) ทั้งก่อนและหลังฉีดน้ำเกลือ มอร์ฟีน (10 มก./กก.) แอสไพริน(150 มก./กก.) หรือสิ่งสกัดจากใบมะม่วงหิมพานต์ ขนาด 62.5, 125, 250, 500, 750 และ 1,000 มก/น้ำหนักตัว 1 กิโลกรัม เข้าทางช่องท้อง ที่เวลา 15, 30, 45, 60, 90, 120, และ 240 นาที โดยเวลาสูงสุดที่อนุญาตให้หนูถีบจักรอยู่บนแผ่นร้อนเท่ากับ 45 วินาทีและนำเวลาที่หนูถีบจักรสามารถทนต่อความร้อนได้มาคำนวณเปอร์เซ็นต์สูงสุดที่หนูถีบจักรสามารถทนต่อความร้อนได้ (%MPE) เพื่อนำมาใช้ในการคำนวณหาพื้นที่ใต้กราฟระหว่าง %MPE และเวลา (area of analgesia) จากการทดลองพบว่า สิ่งสกัดขนาดตั้งแต่ 125 มก./กก. ขึ้นไป สามารถทำให้หนูถีบจักรทนต่อความร้อนได้นานขึ้นอย่างมีนัยสำคัญทางสถิติ และหนูถีบจักรจะสามารถทนต่อความร้อนได้เพิ่มขึ้นตามขนาดของสิ่งสกัดที่สูงขึ้น โดยฤทธิ์ระงับปวดของสิ่งสกัด (500มก./กก.) ถูกยับยั้งได้ด้วยนาล็อกโซน และ NMDA (0.38 มก./กก.) แสดงว่าสิ่งสกัดจากใบมะม่วงหิมพานต์น่าจะออกฤทธิ์ผ่านวิถีของ opioid และ NMDA ในการทดลองที่ทำให้หนูเกิดความเจ็บปวดด้วยความร้อนโดยการส่องไฟที่หางของหนูถีบจักร (tail-flick test) และจับเวลาที่หนูถีบจักรสามารถทนต่อความร้อนได้จนกระทั่งกระดกหางหนี (เวลาสูงสุดที่อนุญาตให้ส่องไฟที่หางหนูเท่ากับ 4 วินาที) พบว่าสิ่งสกัดทุกขนาดที่ใช้ในการทดสอบสามารถเพิ่มเวลาที่หนูทนความร้อนโดยไม่กระดกหางหนีได้นานขึ้นเมื่อเทียบกับกลุ่มควบคุม ในการทดสอบ 7 ครั้งภายในเวลา 4 ชั่วโมงหลังให้สารทดสอบ

ในการทดสอบที่ทำให้หนูขาวเจ็บปวดโดยใช้แรงกดบนอุ้งเท้าหลังของหนูขาว (Randall Selitto test) และเปรียบเทียบน้ำหนักของแรงกดที่หนูขาวสามารถทนได้ก่อนและหลังให้น้ำเกลือ มอร์ฟีน แอสไพริน หรือสิ่งสกัดขนาด 62.5-1,000 มก./กก. เข้าทางช่องท้อง ที่เวลา 15, 30, 60, 90, 120, 240 นาที พบว่าสิ่งสกัดในขนาดตั้งแต่ 250 มก./กก. ขึ้นไปมีฤทธิ์ระงับปวดเมื่อเทียบกับกลุ่มควบคุม ส่วนการทดสอบฤทธิ์ระงับปวดของสิ่งสกัดในหนูขาวที่ทำให้เกิดการอักเสบที่อุ้งเท้าหลังข้างขวาด้วยคาราจีแนน พบว่าเมื่อฉีดน้ำเกลือ มอร์ฟีน อินโดเมทาซิน (5 มก./กก.) หรือสิ่งสกัดขนาด 62.5-1,000 มก./กก. เข้าทางช่องท้องก่อนฉีดคาราจีแนน และทำการทดสอบแรงกดต่ออุ้งเท้าทั้งสองข้างที่หนูขาวสามารถทนได้หลังจากฉีดคาราจีแนน 2 ชั่วโมง พบว่าสิ่งสกัดขนาด 250, 500 และ 1,000 มก./กก. ทำให้หนูขาวสามารถทนต่อแรงกดที่อุ้งเท้าทั้งสองข้างได้เพิ่มขึ้น และฤทธิ์ระงับปวดจะเพิ่มขึ้นตามขนาดของสิ่งสกัดที่เพิ่มขึ้น โดยสิ่งสกัดในขนาด 125, 250 และ 500 มก./กก.ไม่ทำให้หนูขาวสูญเสียการทรงตัวเมื่อเทียบกับกลุ่มควบคุม จากผลการทดลองทั้งหมดสรุปได้ว่า สิ่งสกัดจากใบมะม่วงหิมพานต์มีฤทธิ์ระงับปวด และฤทธิ์ระงับปวดจะเพิ่มขึ้นตามขนาดของสิ่งสกัดที่เพิ่มขึ้นโดยไม่มีผลต่อการทรงตัวของสัตว์ทดลอง และกลไกการออกฤทธิ์ของสิ่งสกัดน่าจะมีความเกี่ยวข้องกับตัวรับของ opioid และ NMDA.

สหสาขาวิชา เภสัชวิทยา ลายมือชื่อนิสิต.....
ภาควิชา เภสัชวิทยา ลายมือชื่ออาจารย์ที่ปรึกษา.....
ปีการศึกษา 2545 ลายมือชื่ออาจารย์ที่ปรึกษาร่วม.....

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JANJUREE KAOPINPRUCK : ANALGESIC EFFECTS OF THE ETHANOLIC EXTRACT FROM ANACARDIUM OCCIDENTALE LEAVES. THESIS ADVISOR: LT. PASARAPA TOWIWAT, PH.D., THESIS CO-ADVISOR: ASSOC. PROF. NIJSIRI RUANGRUNGSI, PH.D.,104 pp. ISBN 974-17-3117-5

The crude extract of *Anacardium occidentale* leaves (ANA) has been used in folk medicine for treating sore gums and toothache. In these studies, we initially determined the analgesic property of a range of ANA doses in the mouse hot-plate test. Hot-plate latencies (cut-off 45 sec) were determined prior to the intraperitoneal (i.p.) administration of 0.9% normal saline solution (NSS), morphine (MO: 10 mg/kg), acetylsalicylic acid (ASA: 150 mg/kg) or various doses of ANA (62.5, 125, 250, 500, 750, 1,000 mg/kg). Hot-plate latencies were subsequently determined at 15, 30, 45, 60, 90, 120, 240 min. The percent maximum possible effect (%MPE) was calculated and used in the determination of the area of analgesia (%MPE-min). ANA in doses of 125 mg/kg and higher produced a significant dose-related analgesic response. ANA (500 mg/kg) produced analgesic response that was naloxone-sensitive and attenuated by NMDA (0.38 mg/kg) suggesting opioid and NMDA-mediated mechanisms. In the mouse tail-flick analgesia test, tail-flick latencies (cut-off 4 sec) were determined in male ICR mice prior to the i.p. administration of NSS, MO, ASA or various doses of ANA (62.5-1,000 mg/kg) and subsequently determined at 7 intervals over a 4-hr period. All doses of ANA produced a dose-dependent analgesic response.

Studies then determined the analgesic effect of ANA using the Randall-Selitto analgesia test. Paw-pressure latencies were determined in male Wistar rats prior to the i.p. administration of NSS, MO, ASA or various doses of ANA (62.5-1,000 mg/kg). Paw-pressure latencies were subsequently determined at 15, 30, 60, 90, 120, 240 min. ANA doses of 250 mg/kg and higher produced a significant dose-related analgesic response. Paw-pressure latencies were also determined in male Wistar rats prior to the i.p. administration of NSS, MO, Indomethacin (5 mg/kg) or various doses of ANA (62.5-1,000 mg/kg). Carrageenan was then administered into subplantar area of a right hind paw to induce inflammatory response. Withdrawal threshold were determined 2 hours after carrageenan administered on both inflamed and non-inflamed paw. ANA doses of 250 mg/kg and higher produced a significant dose-related antihyperalgesic activity. ANA doses of 125, 250, and 500 mg/kg i.p. failed to produce motor impairment compared to vehicle control in rota-rod test. Taken together these results demonstrate that the crude extract of *Anacardium occidentale* leaves produced analgesic effect that was dose-dependent in all analgesic testing models without altered motor performance and mechanism of actions seem to be related to opioid receptor and partially involved NMDA receptor .

Inter-Department of Pharmacology	Student's signature……
Department of Pharmacology	Advisor's signature……
Academic 2002	Co-advisor's signature……

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J. Kaopinpruck

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LIST OF ABBREVIATIONS

μm	= micrometer
m/sec	= metre per second
β	= beta
°C	= degree of celcius
μl	= microlitre
SP	= substance P
α	= alpha
Ca^{2+}	= calcium ion
K^{+}	= potassium ion
%MPE	= percentage of the maximum possible effect
/	= per
min	= minute
cm	= centrimetre
g	= gram
h	= hour
ml/kg	= millilite per kilogram
mg/kg	= milligram per kilogram
i.p.	= intraperitoneal
sec	= second
et al.	= et alii (and other)
N	= sample size
AUC	= area under the curve
L	= litre
LD_{50}	= median lethal dose
ED_{50}	= median effective dose
NMDA	= N- methyl-D-aspatate acid
r.p.m	= round per minute
AUC	= Area under the curve (Area of analgesia)

CHAPTER I

INTRODUCTION

PAIN

The definition of pain according to IASP (The International Association for the Study of Pain) is an unpleasant sensory and emotional experience associated with actual or potential tissue damage, or describes in terms of such damage (Turk and Okifuji, 2000).

Pain is an extremely complex process that involves the interaction of an array of neurotransmitters and neuromodulators at all levels of the neuraxis. Nociception and pain are initiated in the peripheral terminals of primary afferent nociceptors. Nociceptors are activated by multiple factors, including mechanical, thermal, and chemical such as strong acid or base (Bonica, 1990).

Tissue damage results in the release of substances, include potassium, serotonin, histamine, bradykinin, prostaglandins, and substance P. These substances activate peripheral pain receptors which then transmit information to the CNS (Bowsher, 1994).

ASCENDING PAIN TRANSMISSION

There are several ascending pathways in the nervous system for the transmission of nociceptive information . Peripherally, pain sensations begin with free nerve endings in the skin. These peripheral nerves respond to different types of pain stimuli with various different responses depending on the type of sensory fiber to which they are connected. Four main types of sensory axons exist: Aα, Aβ, Aδ and C fibers. These classifications are based on conduction velocities and sizes of the axons. Aα fibers are 13-20 μm in diameter and conduct at 80-120 m/sec, Aβ fibers are 6-12 μm in diameter and conduct at 35-75 m/sec, Aδ fibers are 1-5 μm in diameter and conduct at 5-30 m/sec, and C

fibers are 2.0-1.5 μm in diameter and conduct at 0.5-2 m/sec. Aα, Aβ, and Aδ are myelinated, whereas C fibers are not myelinated (Bonica, 1990; Byers and, Bonica, 2000).

There are two categories of cutaneous nociceptors, the Aδ mechanical and C polymodal. Along with these receptors present in the skin, which are available for activation at all times, there is also a group of nociceptors that for the majority of the time are not available for recruitment, known as "silent" nociceptors. It is believed that this population of receptors must first become sensitized before they can be activated, such as may occur after inflammation, and their activity appears to be associated with hyperalgesia (Bonica, 1990; Byers and, Bonica, 2000).

The Aδ mechanoreceptors are believed not to have free endings in that their fine endings are covered by Schwann cells. Their endings are located in the epidermis of the skin and are activated by mechanical stimuli in most instances. Application of noxious heat to these receptors sensitizes them to heat. This characteristic is also thought to play a role in hyperalgesia. Repeated application of intense mechanical stimuli can decrease the responsiveness of this receptor to mechanical stimuli and abolish it in some instances. Some Aδ nociceptors respond to mechanical, thermal, and chemical noxious stimuli without having been sensitized first and are known as Aδ polymodal nociceptors (Bonica, 1990; Dickenson and Reeve, 1999; Byers and Bonica, 2000).

The C polymodal nociceptor is associated with the unmyelinated C fiber. As the name suggests, these receptors response to a variety of adequate noxious stimuli. There receptive fields are smaller than the Aδ nociceptors, although often the receptive fields of these fibers overlap. Polymodal nociceptors show both adaptation and fatigue, where the response to a repetitive stimulus to the same area can diminish. There are also nociceptors activated by stimuli in the non-noxious range; these progressively respond as the stimulus intensity is increased (Dickenson and Reeve, 1999).

There are also receptors responding to noxious stimuli found in skeletal muscle, joints, and visceral tissue, along with receptors that respond to non-noxious stimuli and convey position, movement, and mechanical forces (Dickenson and Reeve, 1999).

SITE AND MECHANISMS OF SPINAL CORD ANALGESIA

Since analgesia results from depression of transmission of pain stimuli to the brain, and since neural transmission is modified by altering synaptic transmission, the most logical site of analgesia is at the synapses within the “pain” system. The first synapse in pain transmission occurs in the dorsal horn of the spinal cord, where the primary pain fibers which transmit pain sensations from the periphery, synapse with interneurons that transmit pain to the higher centers.

The spinal cord white matter containing axon and the gray matter is divided into 10 laminae. The laminae can be seen as layers of functionally distinct cells that form columns of functionally related cells extending the length of the cord. The different laminae contain different intrinsic cells, and receive different inputs and outputs to the ventral horn and to the brain (Dickenson and Reeve, 1999).

Spinal laminae are not separated by distinct borders. Lamina I forms a rim around the most dorsal part of the gray matter and white matter. This lamina contains large horizontal neurons known as the marginal cells of Waldeyer and historically has been called the marginal zone. These cells have long dendrites that mainly pass over the surface of the dorsal horn, occasionally entering the substantia gelatinosa and forming a vague boundary between the white and gray matter. The dendrites, as well as covering the outer surface of the dorsal horn, also join Lissauer’s tract for up to five or six segments where they join the gray matter again. There are also more smaller neurons located here that may make up the more dorsally located substantia gelatinosa cells. These cells are more prolific in number than the marginal cells. As well as these intrinsic, propriospinal cells, the various laminae also receive afferent inputs. The marginal plexus contains the processes of afferent neurons, superficial neurons, and the processes of deeper cells.

A-δ fibers terminate in lamina I entering via this marginal plexus. Some of the visceral inputs terminate here as well as in laminae II, V, and X.

Laminae II of the spinal cord is also known as the substantia gelatinosa and historically has been called Rolandi substance. The substantia gelatinosa is composed

of lamina II outer (which is mire dorsal and contains densely packed cells) and lamina II inner, which is less compact and more ventrally located.

Hair follicle afferents are the only large-diameter afferents to terminate in lamina II inner. The innocuous C fibers terminate in lamina IIi and the noxious C-fiber terminals are in lamina II outer. However, although C fibers do not terminate in deeper lamina, cells located in laminae V and VI can receive C-fiber inputs onto their dendrites, which extend into more superficial laminae. Afferent terminals from Aδ fibers are few in this lamina, although some have been shown to terminate in lamina II outer.

Laminae III – VI make up the deep dorsal horn. Cells located here have dendrites extending into deeper laminae or into superficial laminae. Inputs arising from cutaneous mechano – and proprioceptors terminate in laminae III and IV. Lamina IV forms the base of the head of the dorsal horn. The cells found here are diffusely arranged, are large, and have dendrites that extend into more superficial laminae so they can receive inputs from afferents terminating more dorsally as well as direct inputs. There are no fine afferents terminating in this lamina. Lamina V forms the neck of the dorsal horn and lamina VI forms the base of the dorsal horn; both receive inputs from thick myelinated fibers. More ventral laminae (VII – IX) have mainly efferents of visceral and somatic motor neurons. Lamina X is located close to the central canal (Dickenson and Reeve, 1999)

The pain fibers (Aδ and C) synapse primarily in the dorsal horn of the spinal cord. Upon entry to the dorsal horn of the spinal cord, the pain fibers travel one to two spinal segments in both directions in a superficial structure called Lissauer' s tracts. Aδ fibers synapse mostly within the outer lamina I and II of the spinal cord. C fibers synapse mostly within lamina I and lamina V. Within the dorsal horn of spinal cord, there are two main types of neurons upon which the Aδ and C fibers synapse. The nociceptive – specific neurons only respond to noxious stimuli, whereas the wide dynamic range neurons respond to innoxious as well as noxious stimuli by increase their firing rate with the increase in stimulus intensity. Wide dynamic range neurons are found in laminae IV, V and VI, and these neurons respond to both Aδ and C nociceptive neurons, Nociceptive-specific neurons are found in lamina I only. Little is known about a third type

of dorsal horn neuron which responds to nociceptive stimuli, the complex neuron found in laminae VII and VIII.

The majority of neurons upon which the peripheral nociceptive neurons (Aδ and C fibers) synapse in the spinal cord project to supraspinal sites via one of three pathways. These three pathways ascend through the anterolateral quadrant (ALQ) of the spinal cord. The spinomesencephalic tract (SMT) projects primarily to the periaqueductal gray of the midbrain. The spinoreticular tract (SRT) synapses in the reticular formation of the pons. The spinothalamic tract (STT) has three projection sites (a; Lateral spinothalamic tract or Neospinothalamic tract b; Medial spinoreticular system or Paleospinothalamic tract c; Anterior/ventral spinothalamic tract). Most STT neurons cross midline around the level of their synapse with the peripheral neurons. These crossing neurons have two project sites: (1) the medial division synapses on the central lateral nucleus of the thalamus, and (2) the lateral division synapses in the ventral posterior lateral nucleus (VPL) of the thalamus. The VPL thalamus is the main pain input to the primary somatosensory cortex. The central lateral nucleus of the thalamus projection to the anterior cingulate gyrus, the basal ganglia, the prefrontal cortex, motor cortex, somatosensory cortex and visual cortex. A minority of STT neurons do not cross midline and project to the ipsilateral intralaminar thalamus. The SRT, SMT and medial portion of the STT are thought to transmit the affective-motivational aspects of pain. The lateral portion of the STT is thought to the transmit the sensory-discriminative aspects of pain (Castro and Zenz, 1991; Dickenson and Reeve, 1999)

There are two other minor pathways which convey nociceptive information from the spinal cord to supraspinal nuclei. Some noxious stimuli are transmitted via the spinocervical tract in the dorsolateral spinal cord to the lateral cervical nucleus. In turn, the stimuli project through the medial lamina to the VPL thalamus. In another pathway, nociceptive neurons project from the dorsal column of the spinal cord to the gracile and cuneate nuclei of the medulla where they synapse on neurons projecting through the medial laminar to the VPL thalamus. Because of their sites of termination, these pathways are also thought to be involved in the sensory-discriminative aspects of pain. Most of the nocciceptive neurons ascending through the STT, spinocervical tract, and dorsal

column-medial laminar pathway project to the contralateral side. The VPL thalamus is part of the ventrobasal thalamic complex (VBT) which is composed of the ventral posterior lateral (VPL) portion and the ventral posterior medial (VPM) portion. The VPL portion receives pain sensation from the trunk and limbs primarily via the STT. The VPM portion receives pain sensation from face via the trigeminothalamic tract (Dickenson and Reeve, 1999).

DESCENDING PAIN INHIBITION

There are three main nuclei responsible for descending inhibition of pain at the spinal cord level. In the medulla, the nucleus raphe magnus (NRM) has serotonergic, noradrenergic and enkephalinergic projections to the ipsilateral dorsal horn of the spinal cord which are inhibitory. These fibers travel through the dorsolateral funiculus and end primarily in lamina I, II and V. In the pons, the locus ceruleus (LC) also known as nucleus A6, has inhibitory noradrenergic projection to the same laminae of the dorsal horn of the spinal cord. The third main nucleus in the midbrain, the ventral periaqueductal gray (PAG) has few direct inhibitory enkephalinergic projection to the spinal cord dorsal horn but mainly acts through excitatory input to the NRM. The NRM projection are thought to be enkephalinergic, so intermediate neurons within the NRM have been postulated as the mechanism converting inhibitory enkepphalinergic input to the excitatory response observed.

The PAG, NRM and LC have been the best defined and most consistently reported pathway of descending pain inhibition. However, there is evidence for several other nuclei and pathways being involved in descending pain inhibition. The NRM combined with the adjacent nucleus reticularis gigantocellularis pars α and the nucleus reticularis paragigantocellularis make up the ventromedian medulla. The nucleus reticularis gigantocellularis which receives direct projection from the spinal cord dorsal horn thought to have its own projection to the NRM, thus activating descending pain inhibition. Also in the medulla, the nucleus of the solitary tract receives input from the NRM and has projections through the dorsolateral funiculus to the spinal cord dorsal

horn. The nucleus of the solitary tract has been suggested as an intermediate partially responsible for the descending pain inhibition produced by the NRM. The only other medullary nucleus though to be involved in descending antinociception is the A1 nucleus which part of the lateral reticular nucleus of the ventrobasal medulla. This noradrenergic nucleus can cause spinal antinociception, but has no direct projections to the spinal cord. It is thought to act through its projections to the NRM.

Within the pons, there are four nuclei responsible for noradrenergic descending pain inhibition in the spinal cord. The first, the LC has already been discussed. The other three, the nucleus subcoeruleus, the A5 nucleus and the A7 nucleus, all have direct noradrenergic projections to the superficial lamina of the spinal cord dorsal horn. Finally, within the mid brain, the nucleus cuneiformis is thought to activate spinal pain inhibition through influence on the NRM.

There is evidence that the three main descending antinociceptive nuclei, PAG, LC and NRM, are interconnected. As described above, the PAG stimulates the NRM, but the NRM also projects to the PAG. The PAG also stimulates and receives inhibitory feedback from the LC. To complete the circuit, the LC causes inhibition of, and receives projection from, the NRM (Castro and Zenz, 1991; Dickenson and Reeve, 1999).

The antinociceptive effects of the serotonergic and noradrenergic projection to the spinal cord horn are effectively blocked by administered opiate antagonist naloxone. Enkephalinergic interneurons within the spinal dorsal horn have been identified. The antinociception projection produced by the noradrenergic and serotonergic projections may be produced by stimulation of the dorsal horn enkephalinergic interneurons (Castro and Zenz, 1991; Dickenson and Reeve, 1999).

Enkephalin interacts with specific opioid receptors which are located on pain-transmitting neurons. The interaction between enkephalin and the opioid receptors depresses transmission of pain information through these neurons. The enkephalinergic neurons synapse with the primary pain fibers and with cells of origin in the spinothalamic tract in laminae V, VI, VII, and VIII. Activation of these neurons during pain depresses pain transmission through the spinal cord.

Non-pain stimuli arriving at the dorsal horn can also activate the enkephalinergic neurons that in turn depress pain transmission. The “gate control theory” postulates that stimulation of peripheral, non-pain-transmitting fibers, activate interneurons that depress transmission of pain sensation within the neuronal network in the dorsal horn. Thus, a tonically active enkephalinergic system could act as a gating mechanism that controls the transmission of the pain sensations to the brain (Melzak and Wall, 1965).

The discovery of opioid receptors in mammals, which when stimulated produced analgesia, and the isolation of the endogenous peptides, such as enkephalin, β-endorphin, and dynorphins, that interacted with the opioid receptors has been very useful in determining the underlying mechanisms of action analgesia (Hughes *et al*., 1975).

It has been suggested that analgesia can result form actions at two sites. One site is at the presynaptic endings of the primary pain fibers, where the activation of the opioid receptors will depress the release of substance P (SP), one of the neurotransmitters involved in the response to pain. It has been well documented that activation (depolarization) of the cell activates Ca^{2+} channels within the cell membrane and results in an increase in intracellular Ca^{2+} that is necessary for the mobilization and release of SP. Any mechanisms that depress the release of SP or interfere with its interaction with receptors on the postsynaptic membrane will depress pain transmission.

The other site is at the interneuronal level within the dorsal horn of the spinal cord. Activation of opioid receptors within this system causes these neurons to be come hyperpolarized, which depresses transmissions of the pain impulse through these neurons (Duggan *et al*., 1981). It has been demonstrated that calcium is also involved in this mechanism. Membrane hyperpolarized occurs as the result of increased K^{+} efflux (conductance) from the cell. The loss of K^{+} causes the interior of the cell become more negative (hyperpolarized), which makes the cell more difficult to activate, resulting in depression of the transmission of the pain impulse through the cell. The efflux of K^{+} from the cell depends upon the release of Ca^{2+} from intracellular storage sites (North and Williams, 1985). Thus, there appears to be two possible sites for analgesia, and both mechanisms involve Ca^{2+}

Calcium is stored within the cell in specialized organelles called the endoplasmic reticulum (ER), which are sometimes referred to as calcisomes (Joseph and Rice, 1989). It has been demonstrated that release of Ca^{2+} results from the interaction between the intracellular transmitter inositol –1,4,5-triphosphate (IP_3) and a receptor on the ER (Meyer *et al*., 1988). Released intracellular Ca^{2+} will increase K^+ conductance out of the cell, resulting in cell hyperpolarized. Calcium and K^+ have a unique relationship in that the IP_3 induced release of Ca^{2+} requires the presence of K^+, yet if K^+ is prevented from leaving the cell by use of K^+ channel blockers, it will further inhibit IP_3 induced release of Ca^{2+}. In addition, the release of Ca^{2+} is self-limiting since Ca^{2+} causes inhibition of the interaction between IP_3 and its receptors on the ER. Therefore, the concentration of intracellular Ca^{2+} controls the rate at which IP_3 is synthesized. In addition, Ca^{2+} enhances the breakdown of IP_3 to IP_4, which is less active than IP_3 in causing the release of Ca^{2+} (Chuang,1989).

Synthesis of intracellular transmitters has been well documented. Cell membranes contain the lipid substance phosphatidylinositol (PIP_2), which is the precursor of IP_3. The synthesis of IP_3 is as follows: Receptors located on the cell membrane are linked to stimulatory proteins called G proteins. Stimulation of the receptor causes a conformational change in the receptor that results in the alteration of the G protein to an active form, which in turn, activates the enzyme phospholipase C. The activation of this enzyme results in the hydrolysis of PIP_2 into IP_3 and diacyglycerol. This reaction has been well documented (Chuang, 1989). It appears that, mechanisms of analgesia that depend upon cell hyperpolarization require the formation and release of the intracellular transmitter IP_3, which causes the release of intracellular Ca^{2+}.

The mechanism of analgesia that depends upon depressed release of SP also involves Ca^{2+}, only through a different action. It has been well documented that an influx of extracellular Ca^{2+} is necessary for the mobilization and release of neurotransmitters. Depolarization of the postsynaptic cell membrane activates the voltage-gated Ca^{2+} channels, resulting in an influx of Ca^{2+} into the cell mechanisms that would limit this influx would be expected to depress the release of SP, thereby depressing transmission of the pain signals (Berridge, 1990; Majerus *et al*., 1986)

There is evidence to suggest that stimulation of opioid receptors, classified as μ receptors, activates the spinal analgesic system. There is a high concentration of opioid receptors within the dorsal horn of the spinal cord, and it has been demonstrated that these receptors are coupled to G proteins. The interaction of enkephalin with the μ-receptors alters the configuration of the G proteins, which results in the release of intracellular Ca^{2+} (Pellegrini-Giampietro *et al*., 1988)

Many recent studies have indicated that the noradrenergic/adrenergic system is also involed in the endogenous analgesic system at the spinal level. There are indication that activations of α_1 and α_2- adrenoceptors, which are found in the dorsal horn, are responsible for this activity. It has been reported that the administration of norepinephrine (NE), the transmitter of the noradrenergic system, into the dorsal horn of the spinal cord suppresses noxious stimuli-induced release of SP. This activity appears to be due to activation of α_2- adrenoceptors. It has also been reported that stimulation of the adrenoceptors activates PIP_2 hydrolysis via interaction with a G protein (Chuang,1989). Therefore, both the opioid and adrenergic system appear to have similar mechanisms at spinal cord level. Some suggest that opioid activation of the spinal analgesic system depends upon activation of the adrenergic system in that drugs such as naloxone that block opioid-induced analgesia do not block noradrenergic - induced analgesia. Conversely, drugs that block the nor-adrenergic system block both noradrenergic and opioid - induced analgesia (Ossipov *et al*., 1987).

The depression of pain at the spinal level appears to be the prime site for analgesia. It has been suggested that analgesia resulting from activation of supraspinal mechanisms activates spinal mechanism and neural pathway that arise in the brainstem nuclei associated with analgesia project to the spinal cord, Where they activate the neuronal system described above. There is little evidence indicating the existence of a neuronal system within the brainstem that will produce analgesia without involving the spinal cord.

GATE CONTROL THEORY

In 1965, Melzack and Wall proposed the gate control theory of pain. The theory eventually obtained widespread acceptance by the scientific and clinical communities because it was an integrated theory which accounted for several unique findings within pain research and practice (Mendell and Wall, 1965a).

The original Melzack and Wall theory of pain is illustrated in Figure 1, A note, impulses evoked by peripheral stimulation are transmitted to three systems: the cells in the substantia gelatinosa, the dorsal column fibers that project toward the brain, and the spinal cord transmission (T) cells that mediate information to the brain. The theory is based on the following propositions: (a) The transmission of nerve impulses from afferent fibers to the spinal cord T cells is modulated by a spinal gating mechanism in the dorsal horn. (b) The spinal gating mechanism is influenced by the relative amount of activity in large-diameter (L) and small-diameter (S) fibers; activity in large fibers tends to inhibit transmission (closes the gate), whereas activity in small fibers tends to facilitate transmission (open the gate). (c) The spinal gating mechanism is influenced by nerve impulses that descend from the brain. (d) A specialized system of large-diameter rapidly conducting fibers labeled the “central control trigger” activates selective cognitive processes that then influence by way of descending fibers the modulating properties of the spinal gating mechanisms (Bonica, 1990; Bonica and Loeser, 2000).

Three years later Melzack and Casey expanded the theory by taking into account subsequently acquired knowledge derived from physiologic and behavioral studies that further emphasize the motivation, affective, and cognitive aspects of the pain experience. These pertain to neural systems beyond the gate and involve interaction of the neospinothalamic and paleospinothalamic projecting systems and neocortical processes. They suggested that the neospinothalamic projecting system in the brain serves to process sensory discriminative information about the location, intensity, and duration of the stimulus, whereas impulses that pass through the paleospinothalamic tract and paramedial ascending system activate reticular and limbic structures that provoke the powerful motivational and aversive drive and unpleasant affect that triggers the organism into action. Neocortical higher central nervous system

processes, such as evaluation of the input in terms of past experience, exert control over both discriminative and motivational systems (Bonica, 1990; Bonica and Loeser, 2000).

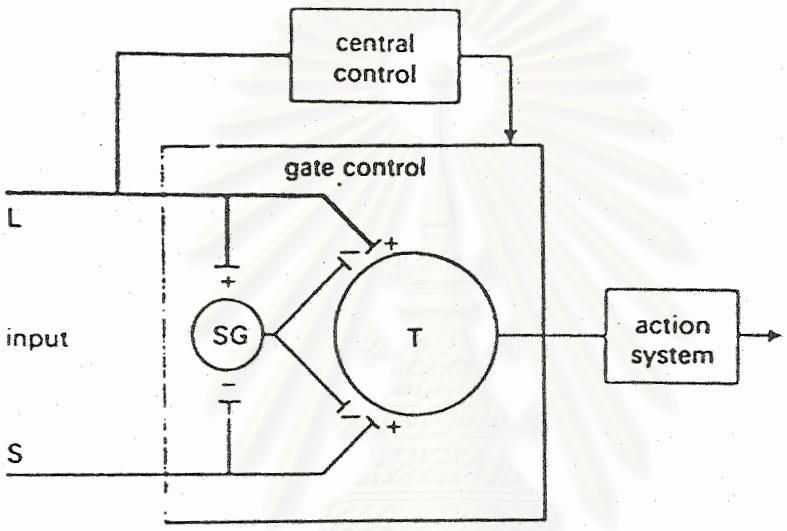


Figure 1: Schematic of the gate-control theory of pain (Mark I). L, large-diameter fibers; S, small-diameter fibers. The fibers project to the substantia gelatonosa (SG) and first central transmission (T) cells. The inhibitory effect exerted by the SG on the afferent fiber terminals is increased by activity in L fibers and decreased by activity in S fibers. The central control trigger is represented by a line running from the large-fiber system to the central control mechanisms; these mechanisms, in turn project back to the gate-control system. The T cells project to the action system. +, excitation; -, inhibition; (From Bonica J. John and Loeser D. John. History of pain concept and theories. In The Management of Pain, USA, 2000)

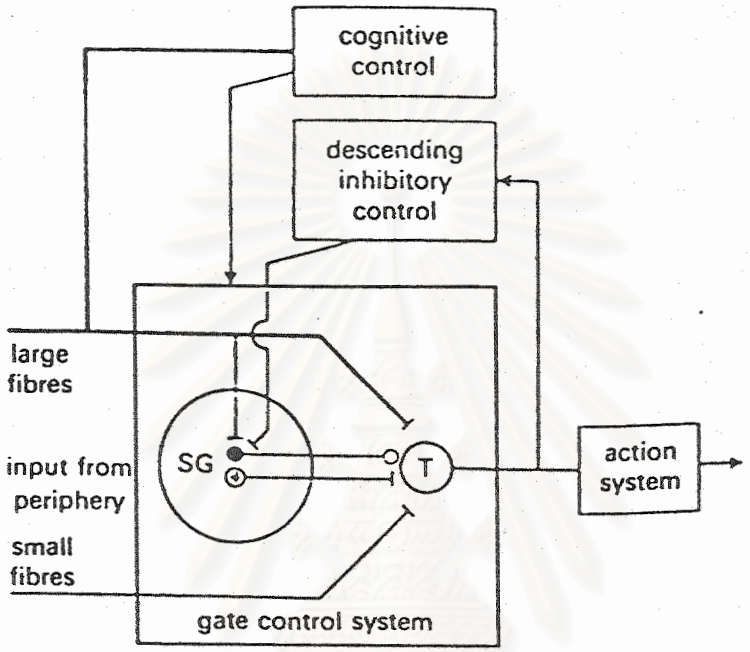


Figure 2: The gate-control theory of pain (Mark II). The new model includes excitatory (white circle) and inhibitory (black circle) links from the substantia gelatinosa (SG) to the transmission (T) cells, as well as descending inhibitory control from brainstem systems. The round knob at the end of the inhibitory link implies that its actions may be presynaptic, postsynaptic, or both. All connections are excitatory, except the inhibitory link from SG to T cell. (From Bonica J. John and Loeser D. John.; History of pain concept and theories. In The Management of Pain, USA, 2000)

In 1982, Melzack and Wall modified their theory to take into account information acquired since the original proposal. The new model is depicted in Figure 2. As may be noted, this model includes excitatory and inhibitory links from the substantia gelatinosa to the transmission cells as well as descending inhibitory control from brainstem systems (Bonica, 1990; Bonica and Loeser, 2000).

MORPHINE ANALGESIA

Opium and its derivatives, such as morphine and codeine, are used primarily for the alleviation or control of acute and chronic pain. However their pharmacological effects are more extensive and include depressant effects on respiration, gastrointestinal motility and some gastrointestinal secretions. These compounds are thought to mimic the activity of endogenous opioid: the enkephalins, endorphins and dynorphins: proenkephalin, pro-opiomelanocortin (from which adrenocorticotropin hormone is also derived) and prodynorphin, respectively. A variety of receptors are involved in the opioidergic system and have been characterized as μ (mu), κ (kappa) and δ (delta) opioid receptors. The endogenous opioids exhibit differential activity at three opioid receptors. The enkephalins and dynorphins exhibit affinity predominantly for δ and κ receptors, respectively, whereas β - endorphin has mixed action on μ and δ receptors. In addition, a separate receptors,ε, has been suggested to exist and is activated by β - endorphin in the mouse vas deferens (Pasternale and Reisine, 1991).

The μ opioid receptor has been further subclassified into two subtypes; the μ_1 opioid receptor and the the μ_2 opioid receptor. The activation of μ_1 opioid receptors in the PAG, the LC and the NRM are thought to be responsible for the analgesic properties of the opioids, while the activation of μ_2 opioid receptors in the brainstem and myenteric plexus may mediate the respiratory depression and the alterations that occur in the gastrointestinal tract. Activation of κ receptors also results in analgesia which is thought to be largely spinally- mediated, and produces a less profound effect on respiratory function. The stimulation of central δ receptors results in analgesia at both spinal and supraspinal sites, while stimulation of peripheral δ receptors inhibits gastrointestinal motility (Reisine and Pasternak, 1991).

MECHANISMS OF OPIOID ACTION

There are three main mechanisms thought to underlie opioid actions. First is presynaptic action whereby opioid receptor activation reduces transmitter release from neuron terminals (tachykinins and excitatory amino acids). This results from the opening of potassium channels (mu and delta receptors) or a closing of calcium channels (kappa), both of which lead to a reduction in calcium ion influx into C fiber terminals, thus diminishing transmitter release. The second mechanism is a postsynaptic hyperpolarization that affect the cell bodies of output neurons, interneurons or dendrites and reduces the evoked activity in neuronal pathway (again via the opening of potassium channels or the closing of calcium channels). Third, there is a disinhibition in circuit of two inhibitory neurons (via $GABA_A$ – mediated disinhibition, or vice versa, or enkephalin neurons in the substantia gelatinosa), in which the second cell is held in check by the other inhibitory neuron. Inhibition of the first the neuron by the opioid allows the second cell to become active, resulting in inhibition of activity. Recent studies of cDNA expression system using cultured cells and frogs' oocytes have shown that the activation of the mu receptor as well as the delta-receptor reduces neurotransmitter release by inhibition of the conotoxin-sensitive calcium channels (Portenoy, 1999; Shipton, 1999).

Transmembrane signal transduction occurs by the sequential interaction of three membrane component: the recognition site (receptor), a pertussis-sensitive G protein and an effector system, which in the case of opioid receptors can be an enzyme (adenyl cyclase or phospholipase C) or ion channel. The agonist binds to the receptor and mobilizes the G protein. The G protein incorporates an inorganic phosphate (GDP is transformed to GTP) and dissociates into subunits (alpha, beta and gamma). The alpha subunits the effector system, while the beta/gamma subunits coordinates stimuli from different pathway. The interaction of the G protein with the receptor determines the ligand affinity, while the duration of dissociation of the alpha from the beta/gamma subunits defines its efficacy. G protein coupled to opioid receptors can alter the activity of enzymes that regulate the intracellular levels of cAMP and inositol 1,4,5-triphosphate (IP_3) and/or directly ion channel conductance. Changes in intracecellular levels of second messengers alter the phosphorylation of intracellular proteins, and short-term

cellular responses occur. These modifications would explain the acute effects of opioid on membrane permeability. Altered protein phosphorylation also cause the induction of nuclear proteins (creb and fos-like transcription factors), Which bind to specific DNA sequences in the promoter region of genes. As a consequence, the rate of generation of these genes increases or decreases. Altered gene expression in target neurons could contribute to the addictive action of opioids (Shipton, 1999).

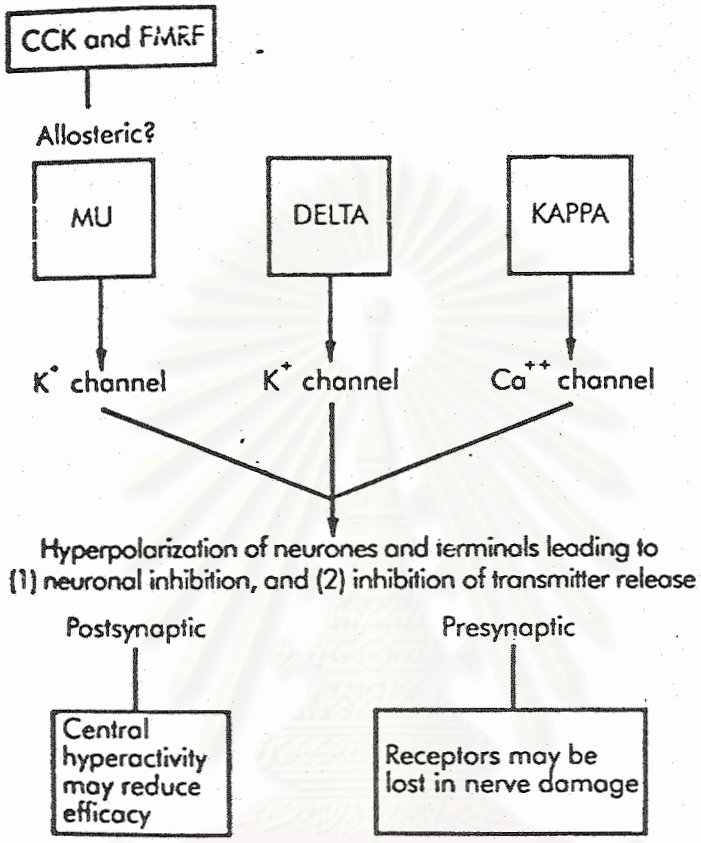


Figure 3 : opioid receptor subtypes and their effector mechanisms. CCK, cholecystokinin; FMRF, FMRFamide (Phe-Met-Arg-Phe-NH_2)-(a neuropeptide for opioid receptor subtypes) ; K^+, potassium ion; Ca^{2+}, calcium ion. (Adapted from: Shipton, E.A. Primary analgesics: the opioids. In Shipton, E.A(Eds.), Pain acute and chronic, 55-75. London: Oxford University Press Inc., 1999).

Recent studies on the spinal opioid systems have begun to address the extent of plasticity in opioid spinal function, which indicated that opioid activity and function are not immutable even to the extent of action of opioid peptide genes by particular stimuli. Most opioid receptors are concentrated around the C fiber terminal zone in lamina 1 and the substantia gelatinosa. Although species difference occur, opioid receptors in the rat spinal cord consist of 70 percent mu, 24 per cent delta and 6 per cent kappa receptors. The proportion of presynaptic sites in the spinal cord varies from 50 to 70 per cent for the subtypes based on drops in levels after rhizotomy. More than 70 per cent of the total mu receptor sites are on the afferent terminal. Mu and delta receptors appear to be coupled in that the activation of one receptor changes the affinity of the other. Both mu and delta opioid receptors can modulate the input of nociceptive information in the dorsal horn. Delta receptors are less effective with visceral pain. Mu ligands include dynorphin and DAGOL (an enkephalin). Opioid can, however, have facilitatory effects on C fiber transmission at low doses (which could explain the itching of the extremities following spinal morphine), while inhibition predominates at higher doses (Shipton, 1999).

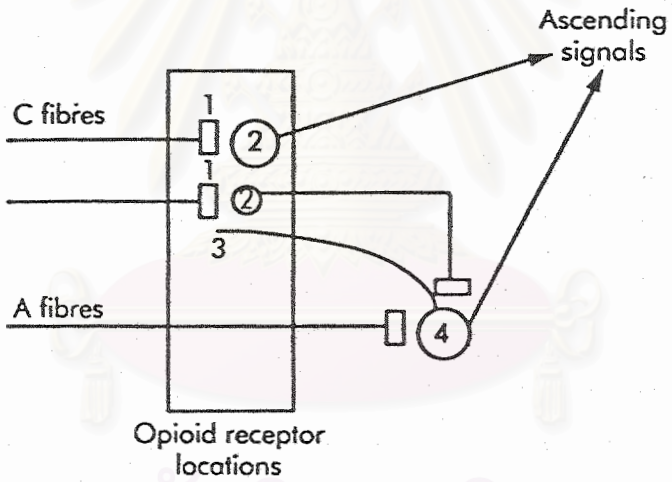
Most mu and delta opioid have similar dose range after intrathecal application, but the most potent opioid are the mu ligands, presumably reflecting the many opioid sites in the spinal cord. The intrathecal combination of a selective mu agonist (DAGOL) and a selective delta agonist (DPDPE) produces marked synergistic effects of the evoked activity of the WDR (wide dynamic range) neurons. The intrathecal application of mu agonist (morphine) and the delta agonist (DADLE) shows little evidence of cross-tolerance. The spinal application of kelatorphan (a peptidase inhibitor) can be reversed by a selective delta antagonist. The inverse relationship between lipophilicity and potency is due to redistribution. Systemic potency ratios are thus poor predictors of spinal effectiveness (Shipton, 1999).

The antinociceptive effect of spinal kappa opioids is unclear. Dynorphin, the endogenous kappa agonist, has some effects that differ from those of typical opioid action. It promotes the action of some neurons and inhibits other nociceptive neurons when applied spinally. The analgesic activity of kappa agonists on kappa receptors may

when applied spinally. The analgesic activity of kappa agonists on kappa receptors may depend upon the intensity of the stimulus rather than its modality. Certainly, all kappa agonists that have analgesic activity in man also have significant mu agonists activity.

The facilitation of spinal opioid (morphine)-induce antinociception by local anaesthetics (bupivacaine) may be associated with a conformation change in the spinal opioid receptors induced by local anaesthetics. All three subtypes of opioid receptors can interact to produce antinociceptive synergy. However, the independent analgesic effects following the activation of non- mu receptors indicates that there is potential for opioid analgesics that delta or kappa agonists (Shipton, 1999).

Non-opioid sigma receptors may be identical to the phenyl cyclohexylpiperidine (PCP) receptor. The PCP receptor appears to be coupled to the NMDA – L – glutamate receptor complex. The NMDA receptor complex may be involved in sustaining or even magnifying pain transmission in the cord.



Opioid sites of action

1. Presynaptic block of transmitter release
2. Inhibition of neurones
3. Inhibition of dendrites
4. Post-synaptic inhibition-non selective

Figure 4 : Sites of opioid action in the spinal cord. (Adapted from: Shipton, E.A. Primary analgesics: the opioids. In Shipton, E.A(Eds.), Pain acute and chronic, 55-75. London: Oxford University Press Inc., 1999).

Human peripheral nerves have been found to contain endogenous opioid ligands (β-endorphin and met-enkephalin) as well as opioid receptors. Opioid receptors have been demonstrated on the peripheral terminals of thinly myelinated and unmyelinated sensory nerves in animals and human. The opioid receptor messenger RNA has been detected in dorsal root ganglia. These finding are in line with functional studies indicating that C fiber neurons mediate the peripheral antinociceptive effects of morphine. Recent animals and human studies reveal that immune cells produce endogenous opioids during inflammation. This production of a matched by an increased expression of different opioid receptors on primary afferent nociceptors, where they can exert analgesic activity. Endogenous ligands of peripheral opioid receptors, opioid peptides (endorphin, enkephalin and dynorphin) and their respective mRNAs have been discovered in inflamed tissue (Shipton, 1999).

SUPRASPINAL ANALGESIA

Supraspinal sites of opioid are now well established and have been localized to areas in the medial brainstem around the nucleus raphe magnus, extending rostrally to the peri-aqueductal and periventricular grey areas. Particularly dense opioid receptor binding is observed in limbic structures, thalamic nuclei and neural areas that control visceral function. Mu, kappa, and delta receptors elicit supraspinal analgesia. The mechanisms of action of opioids at supraspinal levels, in particular how they interact with descending inhibitory controls, are still unclear. It is thought that supraspinal opioids might alter the unpleasant nature of the pain, modulate nociception from the cord by activating descending control systems or interfere with descending controls (which are turned off by noxious stimuli), thus allowing nociception to occur. Nociception might also be modulated by opioids at the thalamic and sensory cortical levels. The simplest situation is that supraspinal opioids increase descending inhibitory influences, these in turn blocking spinal transmission. This is demonstrated by intraventricular morphine reducing the spinal induction of C-fos (a marker of noxious evoked activity). Other findings, however, do not show this direction of effect. Diffuse noxious inhibitory controls are descending controls induced by heterosegmental noxious stimulation, involving both

opioid and serotonergic mechanisms. Morphine reduces these descending controls. The multiplicity of the descending controls (in terms of their locations, pharmacology and spinal projections) forms a framework within which the various directions of effect of opioids can be incorporated. The direct spinal actions of opioids in blocking the spinal transmission of pain would prevent the initial activation of diffuse noxious inhibitory controls and reduce segmental excitations and descending controls (Shipton, 1999).

Supraspinal opioids produce behavioural analgesia. Opioids modulate transmitters involved in other non-nociceptive functions. Respiratory depression, nausea and vomiting may be mediated via opioid receptors in the area of the solitary tract. Dependence may be related to receptor sites in the locus coeruleus and ventral segmental area. Delta- and kappa- mediated analgesia is less associated with respiratory depression, dependence and tolerance than is mu-mediated analgesia (Shipton, 1999).

OPOIOD TOLERANCE

The development of tolerance to opioid analgesia is manifested as a shift to the right of the dose-response curve, or as a decrease in the intensity of the response when a constant dose is repetitively administered. The possibility of rapid dose escalation for pain control is one of the major fears surrounding opioid prescription, regardless of the nature of the pain. At least three factors may be operating simultaneously to cause dose escalation. There may be an increase in the nociceptor stimulus, changes in the receptors or alterations in opioid pharmacokinetics by either self-induction or drug administration, resulting in enhanced elimination (Parkhouse, Pleuvry, and Rees, 1979; Shipton, 1999).

Tolerance to morphine in rodents can be blocked by the NMDA receptor antagonists MK-801, and the oral antitussive dextromethorphan, or by the inhibition of nitric oxide synthase, as well as being attenuated by the use of cholecystokinin antagonists and dynorphin (Shipton, 1999; Reisine and Pasternak, 1991). Cross-tolerance occurs within members of the opiate group, though does not extend to other centrally acting drugs (Parkhouse, Pleuvry, and Rees, 1979).

OPIOID DEPENDENCE

Psychological dependence is hardly ever seen after pain relief with opioids, although physical withdrawal may occur if the dose is suddenly reduced. In mice, pretreatment with pertussis toxin will reduce the incidence of withdrawal signs in animals given chronic high-dose morphine, reinforcing the idea that the development of physical dependence may involve pertussis-sentitive G proteins. Dependence, however, is a combination of the abortion of withdrawal, a psychological preference for the drug-induced state and circumstance. It may be possible to avoid some of the clinical dependence problems associated with the use of mu opioids by using selective ligands for the other receptor subtypes (Parkhouse, Pleuvry, and Rees, 1979; Shipton, 1999).

Addiction is a behavioural pattern of drug use characterized by compulsive self-administration on a continuous or periodic basis in order to experience the psychic effects or to avoid the discomfort associated with its absence. Supplies are usually obtained by deceptive or illegal means. Addiction is extremely rare in cancer patients with no history of drug abuse, and is not a problem with opioid use in an acute pain (Shipton, 1999).

ANALGESIC TESTS

While many methods are used in the testing of analgesic compounds, they are generally different by their ability to differentiate spinally- mediated from supraspinally-mediated analgesia. The methods used to determine spinally - mediated analgesia are more a measurement of reflex activity and include the tail – flick test and the tail-withdrawal test.

TAIL-FLICK TEST

This test was first described over a half century ago (D'Amour and Smith, 1941), and it is still an extensively used test of nociception in rats and mice. At the present time this test of analgesia is a typically modified version of the original D' Amour and Smith. Tail-flick test which measures the latency of a rat withdrawing its tail in response to radiant heat from a light beam focused on the ventral tail surface. In a recent variation of the tail-flick test, an animal is placed in restraint device such that its tail lies flat on a surface. Following the administration of the test compound, a light beam, which serve the heat source, is applied to the tail. Reaction time or response latency, the length of time from application of the light, usually seconds, until the animal remove or flick its tail from the source of heat measured and compared to the animal's pretest time (Prodfit and Anderson, 1975). In general, the mean of 3 baseline latencies (BL) recorded within 30 min prior to any manipulation is compared to test latencies (TL) observed at various times after imposition of some potentially analgesic treatment. The intensity of the radiant heat source is adjusted to yield mean BL of approximately 2.0 s. In order to prevent tissue damage, the heat source is terminated after 6.0 s., cut off time. It's usually between 2 and 10 s (Bars, Gozariu, and Cadden, 2001). The degree of reflex inhibition can then be expressed as a percentage of the maximum possible effect (% MPE) according to the following formula

$$\% \text{ MPE} = [(\text{TL} - \text{BL}) / (\text{cut off time} - \text{BL})] \times 100$$

The reaction time of the tail movement varies with the intensity (power) of the source of radiant heat when it is, more intense, the temperature slope is steeper and, consequently, the reaction time is shorter and the movement is greater. The reaction time varies with the surface area stimulated: when the area increases, the reaction time decreases. Similar findings were obtained when electromyographic responses were recorded in the tail muscles. However, this reaction time also varies with the site stimulated; paradoxically, it decreases when the stimulus is applied to increasingly distal parts of the tail even though the pathway for the afferent signals is longer. Also paradoxically, and perhaps as a result of this, pharmacological data can depend on the part of the tail being stimulated. Thus, it can be shown that the test is more sensitive to morphine when the distal part of the tail is stimulated than when a mire proximal part is stimulated, with the middle part giving an intermediate effect (Bars et al., 2001).

A particular problem with test that use thermal stimulation is the possible influence of the skin temperature. Beecher (1957) has discussed this thoroughly with regard to experiments in humans. In studies, the problem has been largely ignored until recently. It has been reported that changes in the temperature (Beck *et al.,*1974) or blood flow of the skin (Davies and Dray, 1980) alter the effect of cutaneous heat stimulation in electrophysiological studies, and also that the tail – flick latency is affected by the skin temperature (Milne and Gamble, 1989; Tjolsen *et al.,*1989a) since the tail is the most important thermoregulatory organ of the rat (Rand *et al.,*1965). It has been described that the tail-flick occurs when the temperature and the level of the nociceptors in the skin reaches a critical value (Ness and Gebhart, 1986). This concept of critical temperature has been suggested in man (Hardy *et al.,* 1951). It has been stated that the temperature threshold of pain in man seems independent of body temperature pretesting temperature of the skin (Croz *et al.,* 1977).

The time required for heating the tail to a critical response temperature will depend on the initial skin temperature, which is determined by local blood flow (Aukland and Wiig, 1984), within the limits given by deep body and ambient temperatures. Hole and Tjolsen (1990) measured subcutaneous tissue temperature during a radiant heat stimulus and found that the rate of increase in tissue temperature was dependent of

initial skin temperature and the time required to reach a hypothetical threshold temperature . As a consequence, the tail-flick latency is negatively correlated to the ambient temperature (Berge *et al*., 1998) and to skin temperature. The effect of skin temperature on tail-flick latency has been described using radiant heat stimulation (Ren and Han, 1979; Berge *et al*., 1998; Tjolson *et al*., 1989a) as well as with hot water immersion of the tail (Milne and Gamble, 1989). This dependency on the skin temperature may be a source of variation in tail-flick test results, even within the same laboratory. More important is the fact that many experimental treatments affect blood flow and thereby the tail skin temperature (Aukland and Wiig,1984). An increase in tail skin temperature may cause shortened tail-flick latencies and may be interpreted as hyperalgesia. Even skin temperatures that are lower than in untreated animals may occur, and may be interpreted as analgesia. A possible cause of error in the tail-flick test may be due to the effect of repeated testing on the nociception itself, whether due to stress or to local effects in the skin, if the same site is stimulated repeatedly. If the stimulation site is varied along the tail during repeated testing, an error is introduced because tail-flick latencies are shown to vary with stimulation site (Ness *et al*., 1987). A variation of stimulation site would probably complicate the investigation of any temperature effects, because both stimulation site and variations in skin temperature would influence the tail-flick latency, and the relationships would be difficult to accurately interpret.

The potential for treatment-induced vasodilation to cause reduction of the tail-flick latencies is approximately of the same size. Under circumstances when control animals are relatively vasoconstricted, vasodiltaion may lead to and increase in tail skin temperature from about ambient temperature to above 30 °C. The effect of vasodilation is particularly important, as smaller changes in the tail-flick latency have been requires to interpret the results as hyperalgesia rather than as analgesia. Even a modest increase of the mean tail skin temperature of about 3.5 °C due to the lesioning of descending serotonergic systems leads to a reduction of the tail-flick latency from 4-4.5 sec to about 3 sec (Tjolsen *et al*., 1988). If the change in skin temperature was not taken into consideration, a reduction of the tail-flick latency of this size would have been

considered as indication of a hyperalgesic state. Thus, the interpretation of the reduced tail-flick latency observed after transection of the spinal cord. Selective lesions of raphe-spinal serotonergic systems, or administration of 5-HT receptor blocking drugs systemically via intrathecally, has generally been that there exists a tonic descending serotonergic inhibition of spinal nociceptive mechanisms. However, the raphe-spinal 5-HT systems also play a role in vasomotor control, and it seems that the decrease in tail-flick latency previously reported can be completely accounted for by an increase in skin temperature when these systems are functionally impaired (Eide et al., 1988; Eide and Tjolsem 1988; Tjolsem et al., 1988;Eide and Rosland, 1989; Tjolsem et al., 1989).

The relationship between skin temperature and response latency would be expected to vary with different experimental conditions. Obvious factors that might influence this relationship are the mean baseline latency, the site of stimulation, both along the tail, whether dorsal or ventral stimulation is used, and the size of the stimulated area. Properties of the heat source are also likely to be important; whether hot water or radiant heat is used, and whether a step stimulus is applied. To know to what degree the skin temperature is a contributing factor in determining the tail-flick latency under varying conditions, investigations of the effects of all these variables would be needed.

The importance of skin temperature in a particular experiment is difficult to predict since the effect of any manipulation may depend on the vasomotor state of the animal. For instance, administration of a serotonergic antagonist may cause apparent hyperalgesia (Berge *et al*., 1983; Tjolsen *et al*., 1989b) only when there is an active serotonergic input to vasoregulatory neurons. On the other hand, the danger of inducing vasoconstriction and to interpret this as analgesia is greatest when the endogenous activity in these systems is low and there is a relative vasodilation.

It must be emphasized that treatment-induced vasodilation will have the most pronounced effect on tail-flick latency when there is maximal vasoconstriction in the tail. On the other hand, vasoconstrictive treatment or treatment that prevents vasodilation will affect the tail-flick latency only when there is some degree of vasodilation, either due to a relatively warm environment or to arousal, motor activation of stress, The tail-flick latency

will not be affected by treatments that cause vasoconstriction and heat conservation in situations when the temperature of the tail skin is close to ambient temperature.

Stress due to a new environment, handling or injection procedures are factors that may influence peripheral blood flow and tail temperature. Stress with motor activation is liable to cause an increase in heat production, an increase in core temperature and an increased frequency and duration of the periods of vasodiltaion and increase in skin temperature of the tail. It has been shown (Tjolsen and Hole, 1992) that immobilization may cause a considerable increase in core temperature and tail vasodilation, while small doses of morphine (0.5-1 mg/kg) completely abolish such vasodilation.

Conclusively, the tail skin temperature is of significant importance in interpreting the results of the tail-flick test. At least under some circumstances, changes in skin temperature may influence the response latencies. For instance, it is imperative to take the effects of skin temperature into account when investigation factors or using drugs that influence autonomic activity, and thermo- or cardiovascular regulation, such as the monoaminergic systems. The problem may be reduced by recording the tail skin temperature and correcting the tail-flick latency data for changes in the temperature. An alternate method for correction of latencies is to establish the relationship between skin temperature and tail-flick latency in an adequate number of animals under similar experimental conditions, and subsequently to correct tail-flick latencies according to the calculated regression factor (Ren and Han, 1979; Eide and Tjolsen, 1988). This method should be used with caution, because it must be assumed that the experiment is performed under the same conditions as when the correction factor was determined.

Other agents including neuromuscular blockers, skeletal muscle relaxants may have some influence on the tail-flick test since those drugs will prevent animals from removing theirs tails from a heat stimulus when experiencing pain. The results may also be misinterpreted as analgesia.

Recently a group of researchers has modified the mouse tail-flick method for evaluation of the duration of sensory block of local anesthetics (Grant *et al*., 1993). In this model, agents were administered directly at the site of neural communication between

the thermal stimulation and the central processing component of this spinal reflex response. They utilized a 30-gauge needle and administered 20 μl on each side of the tail near the sixth vertebral caudal segment. They demonstrated dose-related responses for assessing the activity of local anesthetic in mice.

HOT-PLATE TEST

The hot-plate technique is often used to determine analgesia that is mediated primarily in the higher centers of the brain (Woolfe and MacDonald, 1944). In contrast to the tail-flick technique, which monitors a spinal reflex (and its central modulation), the hot-plate test is capable of measuring a complex motor response that presumably involves both supraspinal and spinal mechanisms. There are variations of this test which employ different temperatures in order to determine nociception. The 50 °C hot-plate technique, also known as a "cold" plate, utilized in some studies, is used often to determine the analgesic activity of weaker analgesics such as aspirin or nonsteroidal anti-inflammatory drugs. The less consistent results associated with this test, however may deter its use. The 52 °C hot-plate technique, or "warm" plate, measures the analgesic activity of mildly potent analgesics such as morphine and codeine. A hot-plate maintained at 55 °C, is effective when testing potent analgesic compounds including morphine and fentanyl. An elaborate variation of the hot-plate test is the increasing-temperature hot-plate test that has been developed by Tjolsen and colleagues (Tjolsen *et al*., 1991). This test may be used to reliably determine the analgesic potency of weaker analgesics as well as opioids. The starting temperature is 42 °C which is increased at rates that are determined by the investigator, for example 1, 2, 3, 4, 5 or 6 °C/min. The licking of hind paw signals the onset of pain, and this response, once observed by the investigator constitutes the end point. The time of onset and temperature that elicited the response is then recorded.

The hot-plate test is a technique that is applicable to both mice and rats, which involves placing an animal on a preheated (50 °-56 °C) surface (typically an aluminum plate approximately 28 × 28 × 2 cm) that is maintained at specific temperatures by constantly circulating water through passages in the metal plate) and a clear plastic

cylinder, 20 cm in diameter and 30 cm in height is placed on the surface to confine the animal during testing and recording the reaction time to the thermal stimulus or the latency of the first sign of discomfort (vocalization, jumping; or lifting, licking or shaking the paws). Animals are placed onto the platform from an elevation of 5 cm and one of two end points used: the interval between reaching the hot plate and licking of the rear paw or jumping from the surface of the metal plate. In the absence of these behaviors the trial is terminated after a defined time (45 sec is cut off time). The average of the last two baseline latencies (BL) observed within 30 min prior to any treatment is compared to a test latency (TL) recorded at various times after a given intervention. The reduction in responsiveness to noxious stimuli can then be expressed as percent maximum possible effect (%MPE) according to the formula:

$$\% \text{ MPE} = [(\text{TL} - \text{BL}) / (\text{cut off time} - \text{BL})] \times 100$$

The hot-plate test is a commonly used assay of the analgesic efficacy of drugs. In contrast to the tail-flick, it samples highly integrated escape response to noxious stimuli. Analgesic will prolong the reaction time significantly and the dosage required to induce this effect in 50% of the animals (Effective Dose-50, ED_{50}) can be computed.

The hot-plate test is another test of nociception that involves a thermal stimulus besides the tail-flick test. Theoretically, changes in peripheral blood flow may also influence the response latency in this test. This has so far not been properly investigated. However, until such investigations have been performed, one should expect that similar problems may occur in the constant temperature hot-plate test as in the tail-flick test in experiments where peripheral blood flow is influenced by drugs or other experimental manipulations. In the increasing temperature hot-plate test, the influence of peripheral blood flow may be reduced. In this test the temperature of the plate increases gradually from a temperature below pain threshold, and the endpoint is the plate temperature when a hind paw lick occurs, regardless of the time it takes before the response occurs (Hunskaar *et al.*, 1986a). This test therefore will be less, if at all, influenced by the pretest skin temperature. For instance, neurotoxic lesions of serotonergic systems do not induce

an apparent “hyperalgesia” in this test as they do in the constant temperature hot-plate test and the tail-flick test (Bjorkum and Berge, 1988).

PAW-PRESSURE TEST (RANDALL SELITTO TEST)

The paw pressure or paw pinch test measures the average latency of foot withdrawal from a pressure stimulus on the dorsal surface of the rat’s hind paws; using an “Analgesy-Meter” (Ugo-Basile, Comerio-Varese, Italy). The Basile Analgesy-Meter has been designed to perform rapid precise screening of analgesic drugs in the normal and inflamed rat paw according to the Randall-Selitto test (Randall and Selitto, 1957), The instrument is basically a device which exerts a force that increases at a constant rate (a certain number of grams per second). This force is continuously monitored by a pointer moving along a linear scale. The force is applied to the animal’s paw, which is placed on a small plinth under a cone-shaped pusher with a rounded tip. The plinth is made of Teflon, which is biologically inert and has a very low friction coefficient. Thus, if the animal suddenly withdraws its paw, it slips out easily without being injured. The operator depresses a pedal-switch to start the mechanism which exerts the force. When the rat struggles, the operator releases the pedal and reads off the scale the force at which the animal experienced pain. The motor’s speed is constant, thus enabling very reproducible measurements to be made. The motor stops immediately when the foot pedal is released.

Since the screw pitch is 16 mm and the motor speed is 1 r.p.m., the slide moves at a rate of 16 mm per second. The instrument is geometrically designed to increase the force on the paw at a rate of 16 grams per second (16 × 2 with one disc added, 16 × 3 with two, etc.). The rate at which the force increases has been found to be a satisfactory compromise for normal operation. Excessively high speed reduces the duration of the test. But entails a greater degree of error caused by variations in the operators’ s reaction time when the pedal is released. On the other hand, if the speed is too low the animal is restrained for a longer period, which is inconvenient for the operator and may cause the animal to make spurious movements before it actually feels pain.

The applied force is continuously monitored by an indicator moving along a linear scale calibrated in grams × 10 with a pointer riveted to slide, e.g., 12.5 = 125 grams, The scale can be multiplied by 2 or 3 by placing on the slide one or two discs, respectively. For instance, with one disc on the slide, the force will be 250 g, when the pointer indicates 25. With the pointer in the same position, two discs will give a reading of 500 g. The force is sufficient to produce indentations of the glabrous skin of the hind paw but without penetration of the skin. The application of force is stopped when the rat starts to struggle (vigorous attempt to withdraw the paw) to a noticeable degree (whether or not accompanied by shrill vocalization). The paw pressure or paw – pinch thresholds are determined at the withdrawal response. The time at which the rat removes the paw, or struggles to do so, is recorded as the end point. Animals failing to tract to a 500 g force are given scores corresponding to the full scale (500 g). The average of two successive determinations is taken as the latency for paw withdrawal. The animal is gently held during this test but not restrained.

BACKGROUND AND RATIONALE

Acute pain, an unpleasant sensory and emotional experience associated with actual or potential tissue damage as defined by IASP, caused by noxious stimuli including chemical, thermal or mechanical. Thus acute nociception generally elicits a protective or defense response to a noxious stimulus. Inflammatory pain is associated with tissue damage and a resultant inflammatory response. It may be regarded as a mechanism to avoid contact with the area to allow healing to occur; thus there may be an advantage to its presence. It is no longer present once the inflammation has subsided. There are several drugs and methods used for relieving pain which include as following:

1. Analgesic drugs

- Opioids

Opioids are routinely used in postoperative pain and in the treatment of many painful conditions of short-term duration. Morphine, the prototypical opioid analgesic, is the reference standard against which the activity of pain treatment is measured, and morphine serves as the reference for antinociceptive activity in analgesiometric assays for acute nociception. Morphine, an opioid with high potency, is very effective in severe pain but has some serious side effects including drug tolerance, addiction, and respiratory depression.

- Nonsteroidal anti-inflammatory drugs (NSAIDS), including acetylsalicylic acid, indomethacin, and etc. are widely prescribed agents that have been shown to have significant utility in a variety of acute as well as chronic pain states. NSAIDs are structurally diverse but have the common feature in the ability to function as inhibitors of the enzymes cyclooxygenase (COX), the essential enzyme in the synthesis of PGs. Though PGs are normally generated by the constitutive form of cyclooxygenase (COX-1) and serve a number of physiological functions, during inflammation PGs formation is enhanced by the induction of another form of the enzyme i.e. COX-2. NSAIDs owe their analgesic and anti-inflammatory properties to a block of COX enzymes but compounds that select for COX-2 produce analgesia with fewer side effects (Dray,1995).

- Miscellaneous drugs such as NMDA-receptor antagonists (e.g., ketamine), alpha$_2$ adrenoceptor agonist (e.g.,clonidine), beta-adrenergic blokers (e.g., propanolo) and, etc.

2. Acupuncture

3. TENS; Transcutaneous electrical nerve stimulation (Bowsher, 1994).

Analgesic drugs are very effective for relieving pain. Since the prices of the available drugs are very expensive and some drugs might have serious side effects associated with their use, investigation of new drugs from natural products may be useful and reduce these problems. Many parts of trees are never been used, such as leaf, bark, or roots. Therefore, if we can use those parts of the trees to produce new drugs, we might be able to have new drugs at a lower cost.

There are many researches reported about investigating new analgesic drugs from natural products, for example, Otuki *et al*. (2001), presented that ether fraction and triterpene isolated from resin of *Protium kleinii* had an analgesic effect in acetic acid induced writhing and produce significant inhibition of the neurogenic nociception caused by topical injection of capsaicin, but failed to produce analgesic effect in the tail-flick and hot-plate test. In Thailand, there are also several reports about analgesic and anti-inflammatory effects of Thai's herbs. For example, in 2000, Muskaw reported that the extract of *Ochna integerrim Merr*. (called Chang-nao) leaf, twig, stem and bark had an analgesic activity in acetic acid induced abdominal constriction. In Indonesia, Hanani *et al*., (1996) reported that the methanol extract of *Anacardium occidentale* leaves extract showed the analgesic effect when given orally compared with water extract and $CHCl_3$ extract in acetic acid induced abdominal constriction. Preliminary studies performed in our laboratory have shown that orally administration of the ethanol extract of *Anacardium occidentale* leaves in doses of 1,000, 2,000, and 3,000 mg/kg showed the analgesic effect compared with vehicle treated control utilizing mice hot-plate test. These studies are therefore designed to examine in other various animal models the analgesic property of the crude extract from *Anacardium occidentale* leaves in Thailand.

PURPOSE OF THE STUDY

The purpose of this study is to evaluate the analgesic effect of the crude extract from *Anacardium occidentale L*. leaves compared with reference drugs and to investigate the possible mechanisms involved.

HYPOTHESIS

The crude extract from *Anacardium occidentale L*. leaves has analgesic activity in analgesic tests including hot-plate test, tail-flick test, and paw pressure test.

EXPECTED BENEFIT AND APPLICATION

Knowledge from the studies of mechanism and analgesic activity of the crude extract from *Anacardium occidentale L*. leaves may lead to the development of a new analgesic drug from natural sources that never been used for any purposes.

CHAPTER II

REVIEW OF RELATED LITERATURES

ANACADIUM OCCIDENTALE LINN.

Family : Anacardiaceae

Genus : *Anacardium*

Species : *occidentale*

Common name : cashew

The cashew tree, *Anacardium occidentale L*., is a botanical species native of eastern Brazil and was introduced into other tropical countries such as India, Africa, Indonesia, and South East Asia in the 16th century (Santose and Magalhaes, 1999).

The cashew tree (in Thailand called Mamung himaparn) produces many resources and products. The bark and leaves of the tree are used medicinally, and the cashew nut has international appeal and market value as a food. Even the shell around the nut is used medicinally and has industrial applications in the plastics and resin industries for its phenol content. Then there is the pseudo-fruit a swollen peduncle that grows behind the real fruit, which yields the cashew nut. This large pulpy and juicy part has a fine sweet flavor and is commonly referred to as the "cashew fruit" or the "cashew apple." Fresh or frozen cashew fruit concentrate is as common a juice product in South American food stores as orange juice is here (Taylor, 2002).

The cashew nut is defined botanically as the fruit. It grows externally in its own kidney-shaped hard shell at the end of this pseudo-fruit, or peduncle. The nut kernel inside is covered with an inner shell, and between the two shells is a thick, caustic, toxic oil called cardol. Cashew nuts must be cleaned to remove the cardol and then roasted or boiled to remove the toxins before they can be eaten. Native to the northeast coast of Brazil, cashew was domesticated long before the arrival of Europeans at the end of the fifteenth century. It was "discovered" by European traders and explorers and first recorded in 1578; from Brazil it was taken to India and East Africa, where it soon became naturalized. In sixteenth-century Brazil, cashew fruits and their juice were taken by Europeans to treat fever, sweeten breath, and "conserve the stomach" (Taylor, 2002).

The cashew tree and its nuts and fruit have been used for centuries by the indigenous tribes of the rainforest, and it is a common cultivated plant in their gardens. The Tikuna tribe in northwest Amazonia considers the fruit juice to be medicinal against influenza, and they brew a tea of leaves and bark to treat diarrhea (Duke, 1983 and Taylor, 2002). In Guyana uses a bark tea for a diarrhea remedy or colic remedy for infants. Tribes in Surinam use the toxic seed oil as an external worm medicine to kill botfly larvae under the skin. In Brazil a bark tea is used as a douche for vaginal secretions and as an astringent to stop bleeding after a tooth extraction (Taylor, 2002).

Botanist James Duke reports that the fruits bark juice and the nut oil are used for warts, calluses, corns, cancerous ulcer, and even elephantiasis. Anacardol and anacardic acid have shown some activity against Walker carcinosarcoma 256. Decoction of the astringent bark given for severe diarrhea and thrush. Old leaves are applied to skin afflictions and burns (tannin applied to burns is liepatocarcinogenic). Oily substance from pericarp used for cracks on the feet. Cuna Indians used the bark in herb teas for asthma, colds, and congestion. The seed oil is believed to be alexeritic and amebicidal; used to treat gingivitis, malaria, and syphilitic ulcers. Ayurvedic medicine recommends the fruit for anthelmintic, aphrodisiac, ascites, dysentery, fever, inappetence, leucoderma. Piles, tumors, and obstinate ulcers, In the Gold Coast, the bark and leaves are used for sore gums and toothache. Juice of the fruit is used for hemoptysis. Sap discutient, fungicidal, repellent. Leaf decoction gargled for sore throat. Cubans use the resin for cold treatments. The plant exhibits hypoglycemic activity. In Malaya, the bark decoction is used for diarrhea. In Indonesia, older leaves are poulticed onto burns and skin diseases. Juice from the apple is used to treat quinsy in Indonesia, dysentery in the Philippines (Duke, 1983).

In Peruvian herbal medicine today, cashew leaf tea (called casho) is employed as a common diarrhea remedy, a bark tea is used as an antiseptic vaginal douche, and the seeds are used for skin infections. In Brazilian herbal medicine the fruit is taken for syphilis, and as a diuretic, stimulant, and aphrodisiac. A leaf tea is prepared as a mouthwash and gargle for mouth ulcers, tonsillitis, and throat problems, and is used for washing wounds. An infusion and/or maceration of the bark is used to treat diabetes, asthenia, muscular debility, urinary disorders, and asthma. The leaves and/or the bark is also used in Brazil for eczema, psoriasis, scrofula, dyspepsia, genital problems, and venereal diseases, as well as impotence, bronchitis, cough, intestinal colic, leishmaniasis, and syphilis-related skin disorders. North American practitioners use cashew for diabetes, coughs, bronchitis, tonsillitis, intestinal colic, and diarrhea, and as a general tonic (Taylor, 2002).

Cashew its many products, even its "fruit," cover a wide range of uses. In addition to being delicious, it is a rich source of vitamins, minerals, and other essential nutrients (it

has up to five times more vitamin C than oranges and contains a high amount of mineral salts). Volatile compounds present in the fruit include esters, terpenes, hydrocarbons, carboxylic acids, aldehydes, alcohols, ketones, lactones, and an isoprenoid. The fruit is used to make highly-nutritive snacks and juices, and fruit extracts are now being used in body care products. Because of the high amount of vitamin C and mineral salts, cashew fruit is used as a catalyst in the treatment of premature aging of the skin and to remineralize the skin. It is also an effective scalp conditioner and tonic and is often used in shampoos, lotions, and scalp creams for the conditioning activity of its proteins and mucilage (Taylor, 2002).

The different compounds produced from this tree offer a wide range of applications. The bark and leaves of cashew is a rich source of tannins, a group of phytochemicals with documented biological activities. In 1985, these tannin was found to demonstrate anti-inflammatory activity in carrageenan and dextran induced rat paw oedemas, cotton pellet granuloma test and adjuvant induced polyarthritis (Mota, Thomas, and Barbosa, 1985) as well as astringent effects, which may underline cashew's efficacy in treating diarrhea (Taylor, 2002). Anacardic acids are found in cashew; their highest concentration is in the nut shells (Paramashivappa, et.al., 2001). Several clinical studies have shown that these chemicals inhibit tyrosinase activity (Kubo et. al., 1994) (they curb the darkening effect of aging), have molluscicidal properties, and are cytotoxic to certain cancer cells (George and Kuttan, 1997). Cashew's antimicrobial properties were first documented in a 1982 in vitro study. In 1999, another study was published indicating in vitro antibacterial activity of these plant against *Escherichia coli* and *Pseudomonas aeruginosa* (Kudi, et al., 1999). Most recently, a study in 2001 reported that a methanolic bark extract exhibited in vitro antimicrobial activity against 13 of 15 microorganisms tested (Akinpelu, 2001). In 1999, researchers reported that cashew fruit exhibited antibacterial activity against the Gram-negative bacterium *Helicobacter pylori*, which is now considered to cause acute gastritis and stomach ulcers (Kubo, et al., 1999). Its effectiveness against leishmanial ulcers also was documented in two clinical studies. Finally, two studies (one in mice and the other in rats) in 1989 and 1998 document the protective quality of a leaf extract against lab-induced

diabetes: although the extract did not act as hypoglycemic as some others, it did stabilize blood glucose levels near pretest levels (Swanston-Flatt, et al., 1989, and Kamtchouing et al., 1998).

Contraindications: Skin contact with various parts of the fresh plant (leaves, bark, fruit, fruit oil) have been found to cause dermatitis and, otherwise, to react as an allergen. Cashew nuts and apple have also been documented to cause food hypersensitivity reactions (Taylor, 2002).

In 1996, Hanani, et al. reported that the methanol extract of *Anacardium occidentale* leaves extract in dose 1,500 mg/kg and 3,000 m/kg shown the analgesic effect when given orally compared with water extract and $CHCl_3$ extract in acetic acid induced abdominal constriction.

CHAPTER III

METERIAL AND METHODS

ANIMALS

Male ICR mice, weighing 18 - 25 g and male Wistar rats weighing 130-180 g (National Laboratory Animal Center, Salaya, Mahidol University, Thailand) served as experimental subjects. Animals were housed under standard conditions of temperature (25 °C), 12h /12h light / dark cycles and had accessed to standard pellet diet and tap water *ad libitum.* Animals were allowed to acclimate to the facility for at least a week before starting the experiments. At the end of each experiment, animals were sacrificed with chloroform. The number of animals used in each experiment was typically eight to ten. The study protocol had been approved by the Ethical Committee of Faculty of Pharmaceutical Sciences, Chulalongkorn University.

PREPARATION OF THE *ANACARDIUM OCCIDENTALE* LINN. LEAVES EXTRACT

1. Fresh leaves of *Anacardium occidentale* Linn. were collected from Amphur Sriracha, Cholburi, Thailand. All leaves used in this study were collected during November - December 2001.

2. Two kilograms of air dried leaves were dried with oven and crushed to coarsely powder of 580 g.

3. The dried leaves powder was successively extracted with 95% ethanol for 72 h at room temperature. The whole extract (dark-green solution) was filtered and transferred into 3 L conical flask. The filtrate was evaporated to dryness using hot water bath.

4. The yield was ground to powder using mortar and pestle. The powder of *Anacardium occidentale* was kept in tight container at 2-8 °C until the time of animal treatment.

A result from 580 grams of dried *Anacardium occidentale* leaves, crushed and extracted with 95% ethanol was filtered. The filtrate was evaporated resulting in the dry powder of 50 g. Therefore, *Anacardium occidentale* leaves contained approximately 12% w/w yield of evaporated *Anacardium occidentale* powder. The powder appeared dark-green color.

The dark-green powder of the extract was dissolved in 0.9% normal saline and the fine suspension of the extract was used for the pharmacological study. The doses employed are expressed in terms of dried powder (mg/kg body weight).

DRUGS

The *Anacardium occidentale* Linn. leaves extract and reference drugs were dissolved or suspended in 0.9% sodium chloride solution (The Government Pharmaceutical Organization, Thailand) and given intraperitoneally to the animals. The control animals were given an equivalent volume of vehicle in the same route. Morphine sulphate (10 mg/kg, Thai FDA), acetylsalicylic acid (150 mg/kg, Sigma Chemical Co., USA), and indomethacin (5 mg/kg, Sigma Chemical Co., USA) were used as standard analgesic drugs. Naloxone (1 mg/kg, Sigma Chemical Co., USA) and NMDA (0.38 mg/kg, Sigma Chemical Co., USA), carrageenan (1 % in 0.9 % sodium chloride solution 50 μl/rat, Sigma Chemical Co., USA).

EXPERIMENTAL METHODS

Hot-plate Analgesic Testing

The male ICR mice weighing 18-25 g were used. Analgesic testing was determined using the hot-plate method. The surface of the hot plate (measuring 28×28 cm was set at $50\ ^{\circ}C \pm 0.5\ ^{\circ}C$ and was surrounded by a clear Plexiglas wall cylinder, 20 cm in diameter and 30 cm in height to confine the animal to the heated surface during testing. On the day of testing, animals were randomly assigned to one of eight treatment groups and underwent 3 pre-drug baseline trials on the hot-plate spaced 5-10 min apart. Only those animals which had a pretreatment hot-plate latency time of less than 45 sec were utilized in these studies. Mice were then administered various doses of treatments and retested. Each mouse was placed on the hot-plate from an elevation of 5 cm and the latency to the licking of a rear paw or a vigorous jumping up from the surface of the metal plate was used as the end point and recorded with a stopwatch. If this behavior was not observed within 45 sec the animals was removed from the hot-plate, given a score of 45 for its paw-lick latency and returned to its cage (the maximum time allowed for an animal to remain on the surface of the plate during testing was 45 sec). The average of the last two trials served as the baseline pre-drug paw-lick latency.

Immediately, after the third baseline trial on the hot-plate, the drug administration took place with intraperitoneal (i.p.) vehicle (10 ml/kg) or test drugs, morphine sulphate (10 mg/kg), acetylsalicylic acid (150 mg/kg) or various doses of *Anacardium occidentale* leaves extract (ANA; 62.5, 125, 250, 500, 750, and 1,000 mg/kg). All animals were placed on the hot-plate for 7 subsequent trials at 15, 30, 45, 60, 90, 120, and 240 min after injection. Thus, ED_{50} were computed and dose- time response curve were generated.

Mouse Tail-flick Analgesic Testing

These studies employed the tail-flick assay described by D'Amour and Smith (1941), with minor modifications. Male ICR mice weighing 18-25 g were used (n = 10 per group). Mice were placed in individual Plexiglas restrainers with an opening to allow the tail to protrude. Each tail rested in a shallow groove housing a light sensitive sensor. A beam of radiant heat (24-V, high amperage 150- watt light bulb situated 8 cm above the tail) was aimed at the middle of the marked dorsal portion of the distal part of each subject's tail that has been blackened length 1 cm with a black ink maker pen in order to absorb the maximum amount of heat and for uniform heat absorption (about 4 cm from the tip). The device (Harvard Tail-flick Analgesia meter) automatically recorded (in 0.1 sec) the latency between the onset of the light beam stimulus and the response to heat, at which point the light beam was terminated. The maximum duration of each test was set at 4.0 sec to minimize the potential for thermal injury. The stimulus intensity was set so that the baseline tail-flick latencies were approximately 1.0 -1.5 sec (intensity 3.7 A). The intensity was not changed for any animal within any given experiment. Animals failing to respond within 1.5 sec were excluded from testing. On the day of testing, all animals were test 3 predrug tail-flick baseline trials conducted at 10-15 min intervals. The score from the third trial served as the baseline measure for each subjects.

Immediately, after the third baseline trial on the tail-flick test, the drug administration took place with either intraperitoneal (i.p.) vehicle or test drugs morphine sulphate (10 mg/kg), acetylsalicylic acid (150 mg/kg) and ANA (62.5, 125, 250, 500, 750, and 1,000 mg/kg). Tail-flick latencies were recorded at 15, 30, 45, 60, 90, 120, and 240 min after injection. Thus, dose-and time response curve were generated. Analgesic will prolong the reaction time significantly and the doses required to induce this effect in 50% of the animals (Effective Dose-50, ED_{50}) can be computed.

Paw Pressure Test (Randall Selitto Test)

Male Wistar rats weighing 130-180 g were used in this test with an Analgesymeter (Ugo Basile, Milan) a force increasing at a constant rate was applied to the rat's hind paw by placing the animal's paw on a small plinth under a cone-shaped pusher with a round tip. The applied force was continuously monitored by an indicator moving along a linear scale calibrated in grams. The Analgesymeter was fitted with a single weight such that the maximum pressure exerted on the paw was 500 g. The pressure on the paw was increased at a constant rate of 32g/sec. The application of force was stopped when the rat started to struggle (vigorous attempt to withdraw the paw) to a noticeable degree (whether or not accompanied by vocalization), the paw pressure threshold was determined at the withdrawal response. The time at which the rat removed the paw, or struggled to do so, was recorded as the endpoint. Animals failing to react to a 500 g force were given scores corresponding to the full scale (500g). The animal was held during this test but not restrained. On the day of testing, all animals underwent 3 pre-drug paw pressure baseline trials conducted at 10-15 min intervals. The score from the last two trials served as the baseline measure for each animal.

Immediately, after the third baseline trial, the drug administration took place with either intraperitoneal (i.p.) vehicle or test drugs morphine sulphate (10 mg/kg), acetylsalicylic acid (150 mg/kg) and *Anacardium occidentale* leaves extract in dose 62.5, 125, 250, 500, 750, and 1,000 mg/kg. All animals were test for the paw pressure threshold test for 6 subsequent trials at 15, 30, 60, 90, 120, and 240 min after injection. Thus, dose-and time response curve were generated.

Carrageenan-Induced Inflammatory Hyperalgesia Testing

Male Wistar rat weighing 130-180 g (n = 8 per group) were used in this experiment using the model described by Hargreaves *et al*. (1998). On the day of testing, all animals were pretreat with either i.p. vehicle or test drugs (Santos et al., 1999; Mendess et al., 2000; Juhanek et al., 2001; Otuki et al., 2001) and 30 min later the plantar surface of the right hind paw in each rat was injected with 50 μl of 1% carrageenan and returned to its cage for resting. Two hours after carrageenan injection (Hargreaves, K., 1988; Megaraughty et al., 2001), animals were tested for mechanical hyperalgesia with an Ugo Basile Analgesymeter. Using a wedge-shaped probe, an increasing weight was applied to the paw (750 g maximum) and the withdrawal threshold determined as the first sign of a pain response. Each animal's withdrawal threshold was an average of two trials, which were separated by at least 10 min. The left hind paw was not injected with carrageenan but was similarly allowing direct comparisons between inflamed and non-inflamed paws for each animal.

Analysis of the analgesic mechanism of action of ANA

The possible participation of the opioid system in the antinociceptive effect of ANA was investigated. To analyze this mechanism we also used the model of mice hot-plate test. Animals were pretreated with naloxone 1 mg/kg i.p. (Pieretti et al., 1999; Perrot et al., 2001) and NMDA 0.38 mg/kg i.p. (Davis and Inturrisi, 1999) 30 min before ANA (500 mg/kg i.p.) administered.

Rota-rod test

To rule out the possibility of motor impairment from intraperitoneal administration of *Anacardium occidentale* leaves extract, mice were tested for their ability to perform a rota-rod test after administration various doses of ANA (n=6) compared to vehicle controls intraperitoneally. Mice were placed on a horizontal rod (3.5 cm diameter) rotating at a speed of 16.5 rpm (Dunham and Miya, 1957). The mice capable of remaining on the top for 3 min or more, in three successive trials were selected for the study (Skyba et al., 2002; Chattopadhyay et al., 2003). Each group of the animals was then placed on the rod at an interval of 30, 60, 120, and 240 min after injection. The results are expressed as the time (seconds) in which animals remained on the rota-rod. The cut-off time used was 180 min (Vaz et al.,1996).

TOXICITY TEST AND LD_{50} DETERMINATION

An acute toxicity study was done by determining LD_{50}, calculated from the lethality within 72 h after intraperitoneally administration of various doses of the crude extract of *Anacardium occidentale* leaves by Litchfield and Wilcoxon (1949) method.

DATA TREATMENT AND STATISTICAL ANALYSE

Statistical analyses were performed on the dose-response curves by analysis of variance (ANOVA) and, where appropriate, were followed by Newman-Keuls' *post hoc* testing (Tallarida and Murray, 1987). The time-course of hot-plate latency, tail-flick latency and paw-pressure threshold are expressed as the mean percent maximum possible effect (%MPE) according to the following formula:

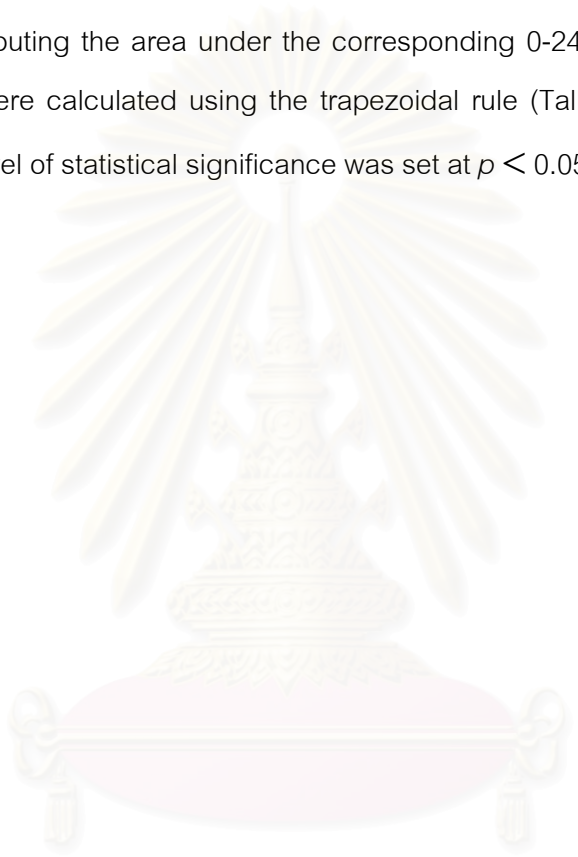
$$\%MPE = \frac{\text{drug latency} - \text{predrug latency}}{(\text{cut-off time}) - \text{predrug latency}} \times 100$$

Note: cut-off time for hot-plate test = 45 seconds

Cut-off time for tail-flick test = 4 seconds

Cut-off time for paw pressure test = 750 g

Dose-effect curves for the hot-plate, tail-flick and paw pressure assays were derived by computing the area under the corresponding 0-240 min time-course-%MPE curves; areas were calculated using the trapezoidal rule (Tallarida and Murray, 1987). The minimum level of statistical significance was set at $p < 0.05$.



CHAPTER IV

RESULTS

MOUSE HOT-PLATE TEST

To demonstrate the validity of the hot-plate analgesic testing following intraperitoneal (i.p.) drug administration, mice received morphine sulphate (MO; 10 mg/kg) i.p. and were tested during the subsequent 240 min period. As expected MO significantly (p<0.01) increased hot-plate latency producing an area of analgesia of 15,583.17±2,113.01 %MPE-min compared with that of normal saline solution (NSS) (501± 96.35 %MPE-min; Figure 5). The i.p. administration of acetylsalicylic acid (ASA; 150 mg/kg), a nonsteroidal anti-inflammatory drug (NSAIDs), also influenced the hot-plate latency and area of analgesia when compared to NSS (p<0.01; Figure 6).

Initial studies utilizing the hot-plate test in mice to examine the efficacy of the crude extract of *Anacardium occidentale* leaves (ANA) in producing analgesia. Mice were then administered NSS or various doses of ANA (62.5, 125, 250, 500, 750, 1,000 mg/kg) i.p. ANA doses of 125 mg/kg or higher produced significant (p<0.05, p< 0.01, p< 0.01, p< 0.01, respectively) analgesic responses compared to NSS. Additionally, ANA doses of 125 mg/kg or higher also produced significant (p<0.05, p< 0.05, p< 0.01 p< 0.01, p< 0.01, respectively) analgesic responses when compared to the lowest dose of ANA used (Figure 7). MO showed the highest analgesic response compared to all test groups. ANA dose 125 mg/kg produced analgesic response similar to ASA (Figure 8).

When the log of the ANA dose was plotted versus the area of analgesia a significant linear correlation was observed. When all six doses of ANA (62.5, 125, 250, 500, 750, 1,000 mg/kg) were plotted a significant linear correlation coefficient (r^2) equal to 0.87 was observed, while the plotting of only four doses (125, 250, 500, 750 mg/kg) revealed a significant linear correlation coefficient of 0.98 (Figure 9& 10). The analgesic

peak effect of ANA was reached within 45 min after i.p. administration in all ANA doses tested and individual time courses of the responses are shown in Figure 11.

In order to investigate any role of the opioid receptor in ANA actions, mice were then administered i.p. NSS, naloxone (NAL; 1 mg/kg), an opioid receptor antagonist, ANA (500 mg/kg) or the combination of naloxone and ANA (1/500 mg/kg). Naloxone alone failed to produce significant responses when compared to vehicle control. ANA produced a significant ($p<0.01$) responses when compared to vehicle control. The inclusion of naloxone significantly ($p<0.01$) attenuate the analgesic response due to ANA indicating that opioid receptors are most likely involved in the analgesic response produced by ANA (Figure 12).

To further explore the mechanism of ANA in this analgesic testing model, mice were then administered i.p. NSS, N-methyl-D-aspartic acid (NMDA; 0.38 mg/kg), ANA (500 mg/kg) or the combination of NMDA and ANA (0.38/500 mg/kg). NMDA alone failed to produce significant analgesic responses when compared to vehicle control. ANA produced a significant ($p<0.01$) analgesic response when compared to vehicle control. The inclusion of NMDA attenuated the ANA response ($p<0.05$) when compared to ANA alone indicating that NMDA receptor complex is partly involved in the analgesic response produced by ANA (Figure 13). ED_{50} was calculated from the log dose probit line and was equal to 363.08 mg/kg (Figure 14).

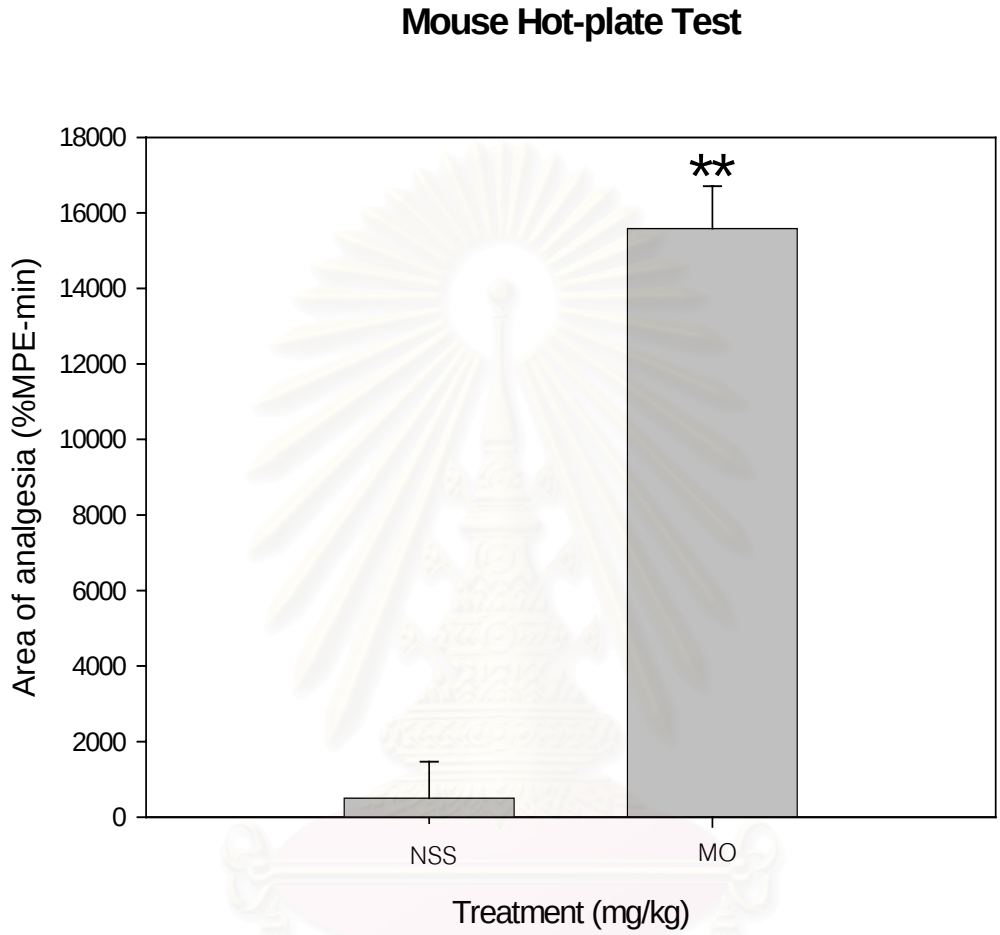


Figure 5 Area of analgesia (%MPE-min) from 0-240 min after intraperitoneal administration of 0.9% normal saline solution (NSS) and morphine sulphate (MO; 10 mg/kg) N=10 for all groups. ** $p < 0.01$ significantly different compared to NSS.

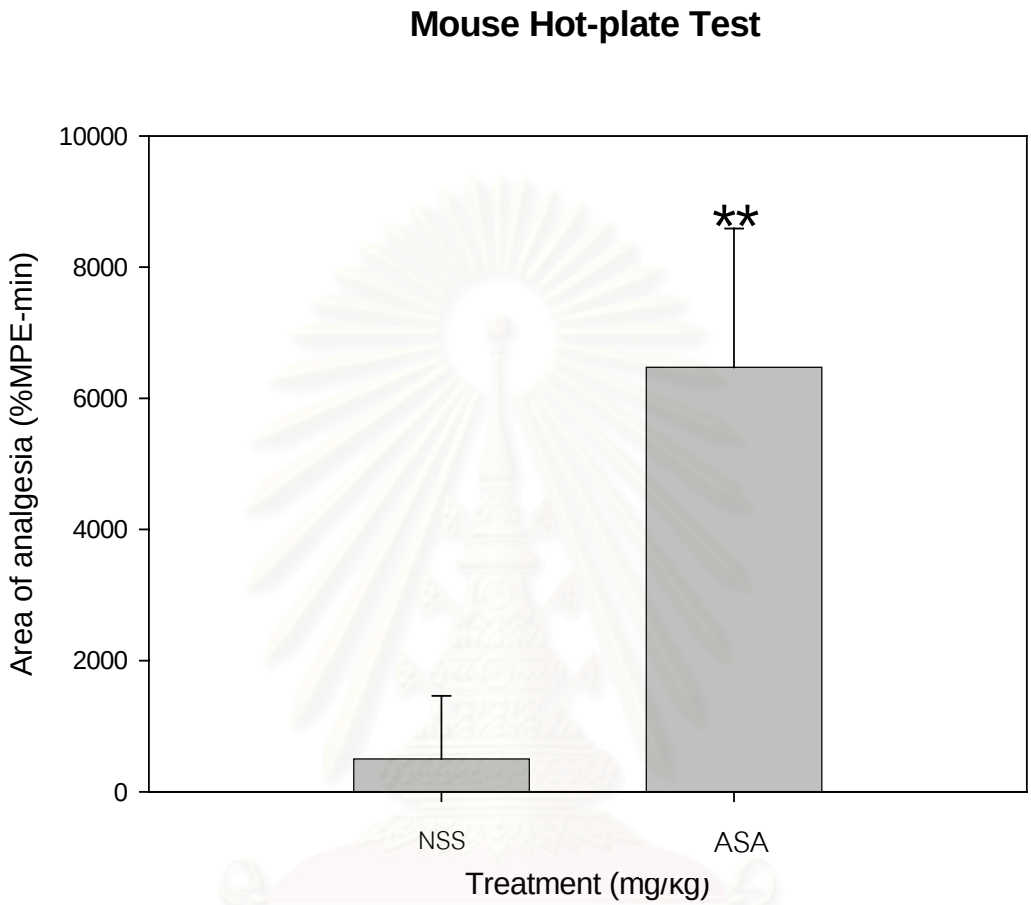


Figure 6 Area of analgesia (%MPE-min) from 0-240 min after intraperitoneal administration of 0.9% normal saline solution (NSS) and acetylsalicylic acid (ASA; 150 mg/kg) N=9 for all groups. ** $p < 0.01$ significantly different compared to NSS.

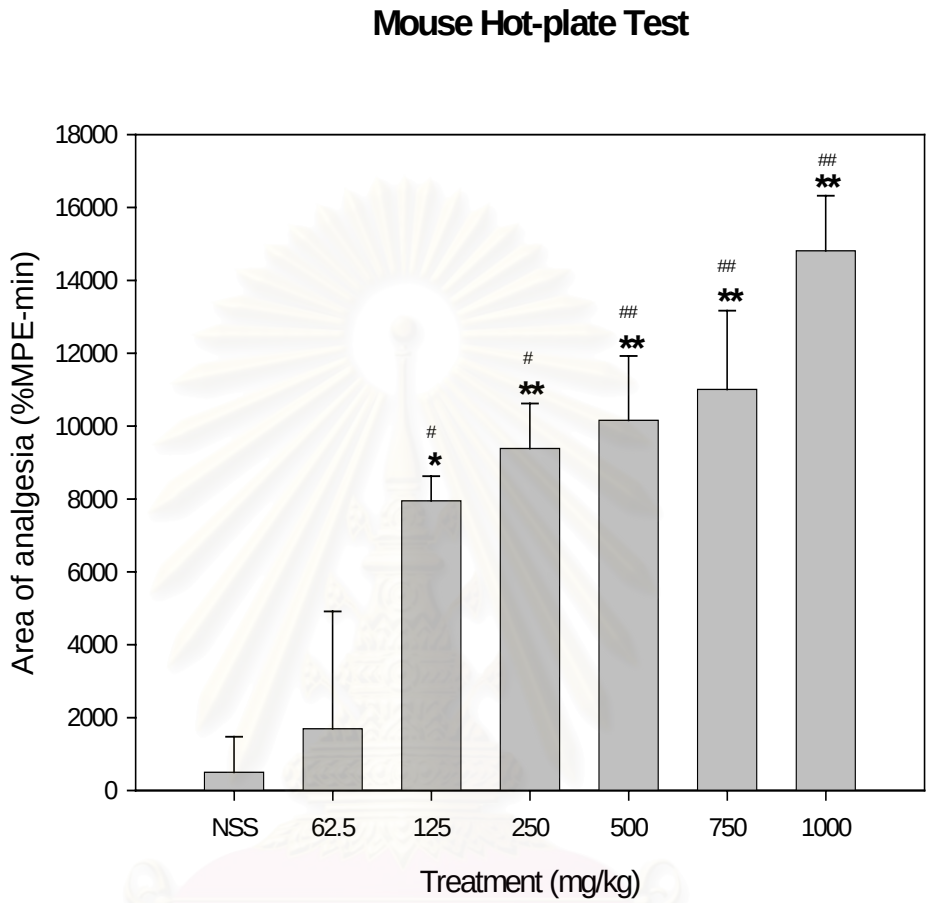


Figure 7 Area of analgesia (%MPE-min) from 0-240 minutes after intraperitoneal administration of 0.9% normal saline solution (NSS) and various doses of ANA (62.5-1,000 mg/kg). N=10 for all groups. *$p < 0.05$, ** $p < 0.01$ significantly different compared to NSS, # $p < 0.05$, ## $p < 0.01$ significantly different compared to ANA 62.5 mg/kg.

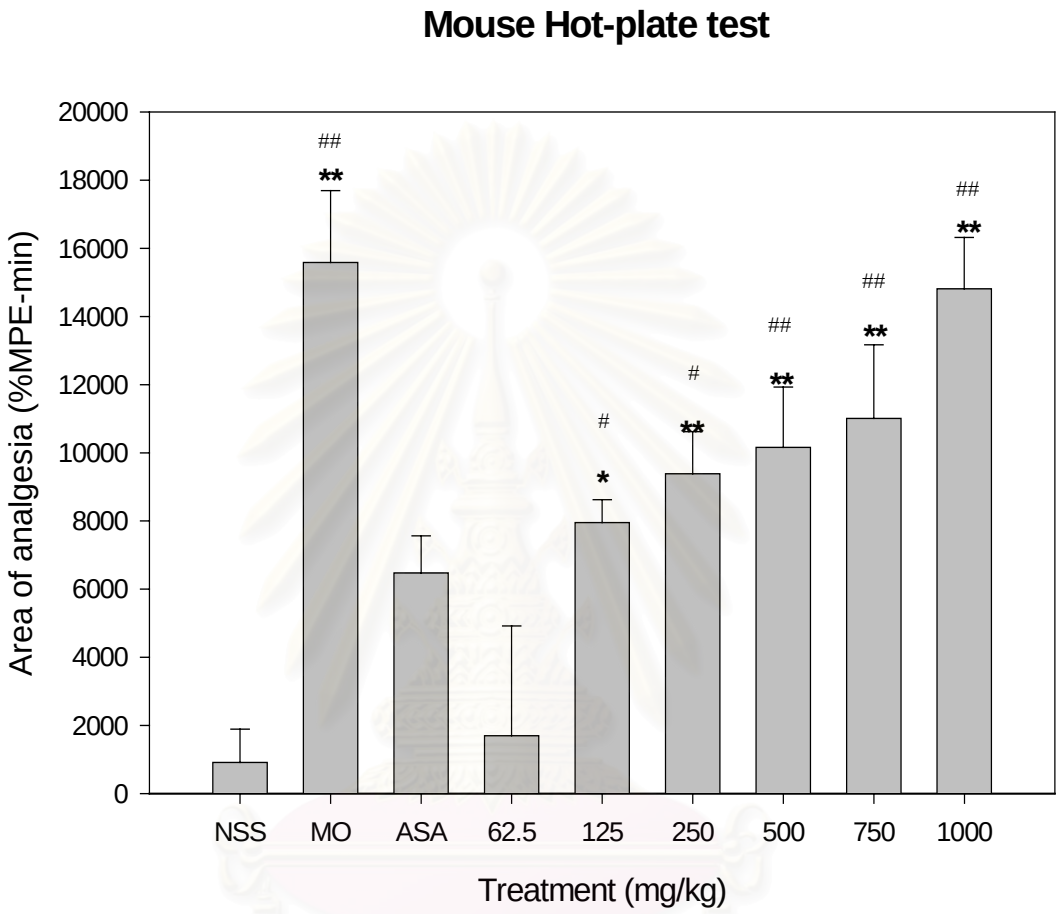


Figure 8 Area of analgesia (%MPE-min) from 0-240 minutes after intraperitoneal administration of 0.9% normal saline solution (NSS), MO (10 mg/kg), ASA (150 mg/kg), and various doses of ANA (62.5-1,000 mg/kg). N=10 for all groups. *$p < 0.05$, ** $p < 0.01$ significantly different compared to NSS, # $p < 0.05$, ## $p < 0.01$ significantly different compared to ANA 62.5 mg/kg.

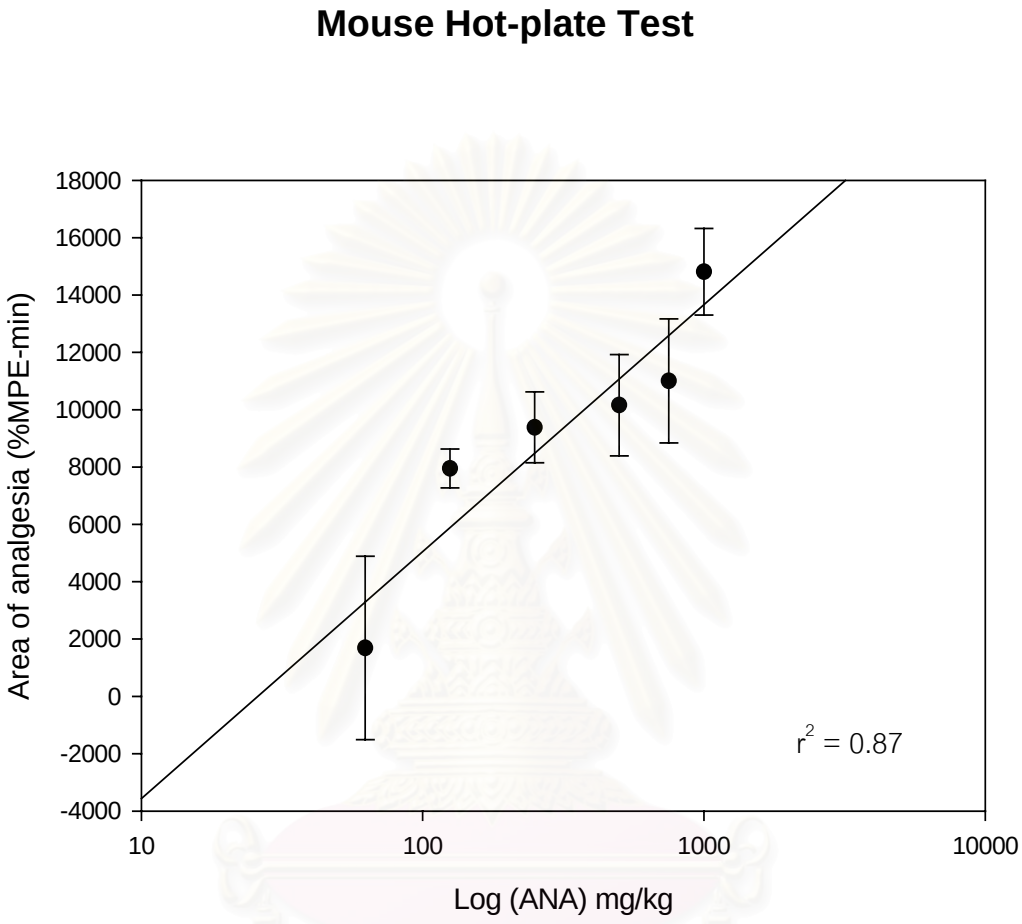


Figure 9 Linear regression of area of analgesia (%MPE-min) from 0-240 minutes after intraperitoneal administration of *Anacardium occidentale* leaves extract (ANA; 62.5-1,000 mg/kg). N = 10 for all groups. The regression equation was Y = 2158.5X + 1612.6, r^2 = 0.87

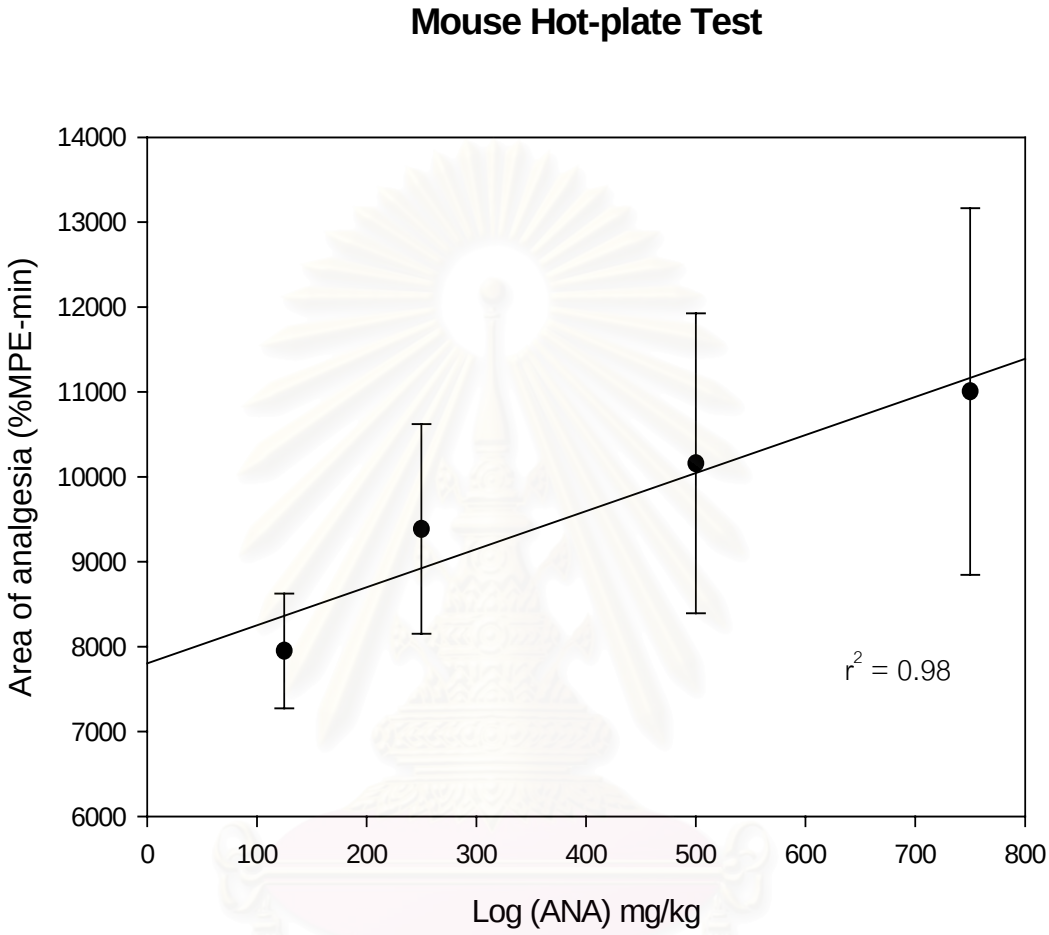


Figure 10 Linear regression of area of analgesia (%MPE-min) from 0-240 minutes after intraperitoneal administration of *Anacardium occidentale* leaves extract (ANA; 125-750 mg/kg). N = 10 for all groups. The regression equation was Y = 994.37X + 7139, r^2 = 0.98

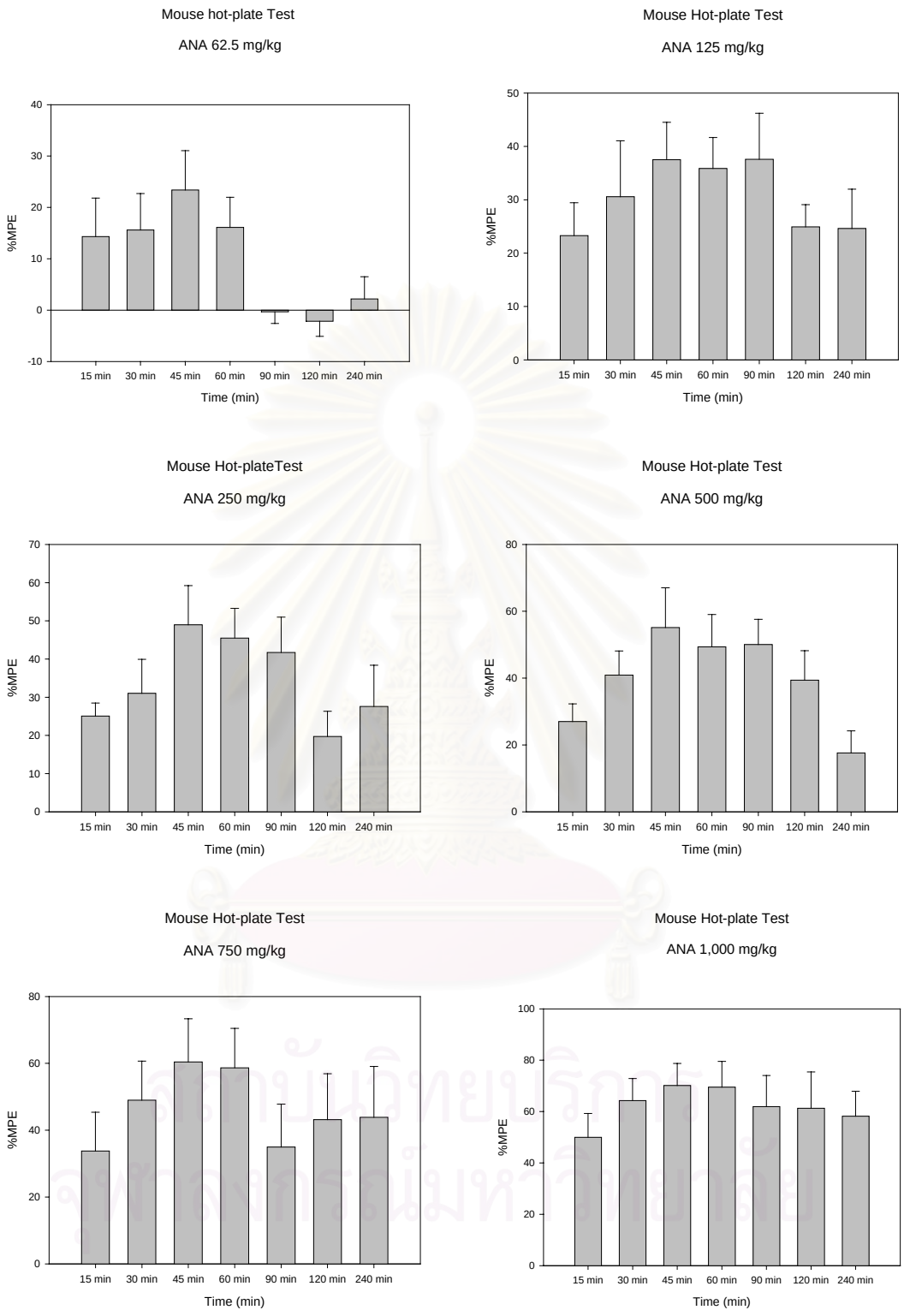


Figure 11 Individual time courses of the response (%MPE versus time (min) after intraperitoneal administration of various doses of ANA (62.5-1,000 mg/kg). N=10 for all groups.

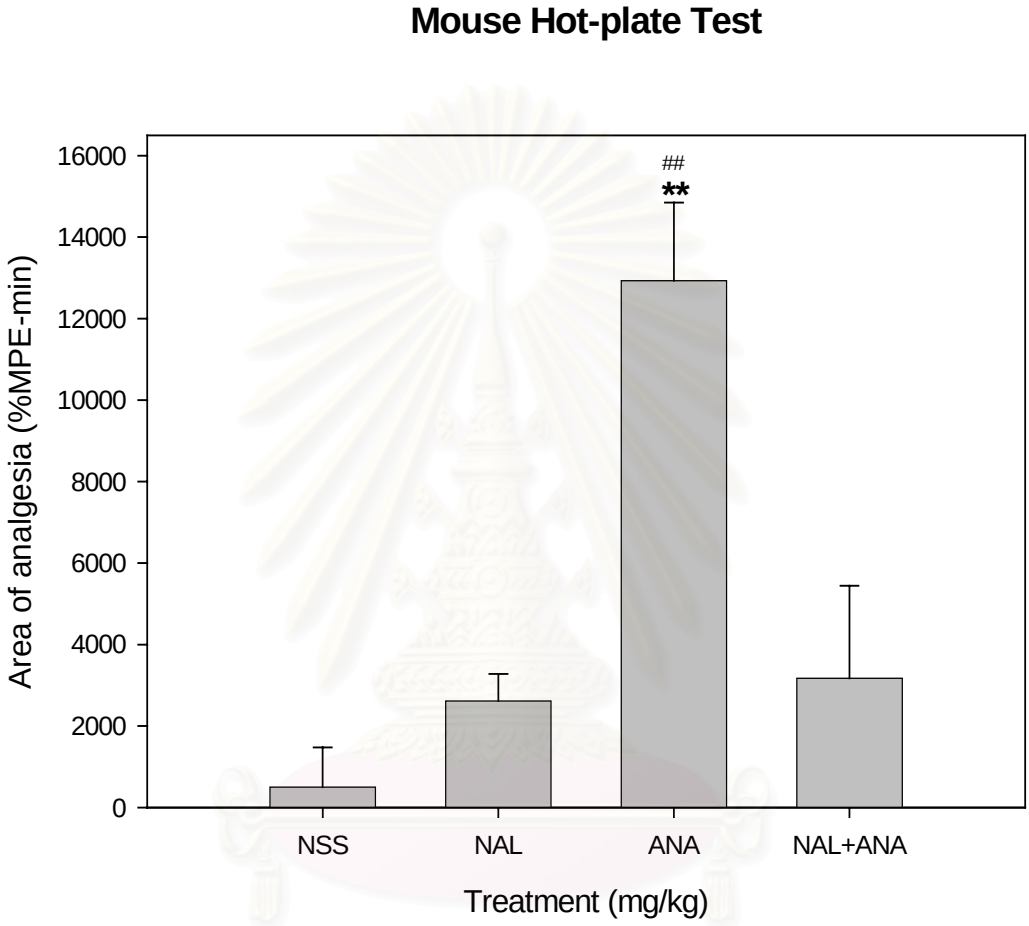


Figure 12 Area of analgesia (%MPE-min) from 0-240 minutes after intraperitoneal administration of 0.9% normal saline solution (NSS), ANA (500 mg/kg), Naloxone (NAL; 1 mg/kg), and NAL+ ANA (1/500 mg/kg) N=10 for all groups. $^{**}p < 0.01$, significantly different compared to NSS, $^{\#\#}p < 0.01$, significantly different compared to NAL and NAL+ANA.

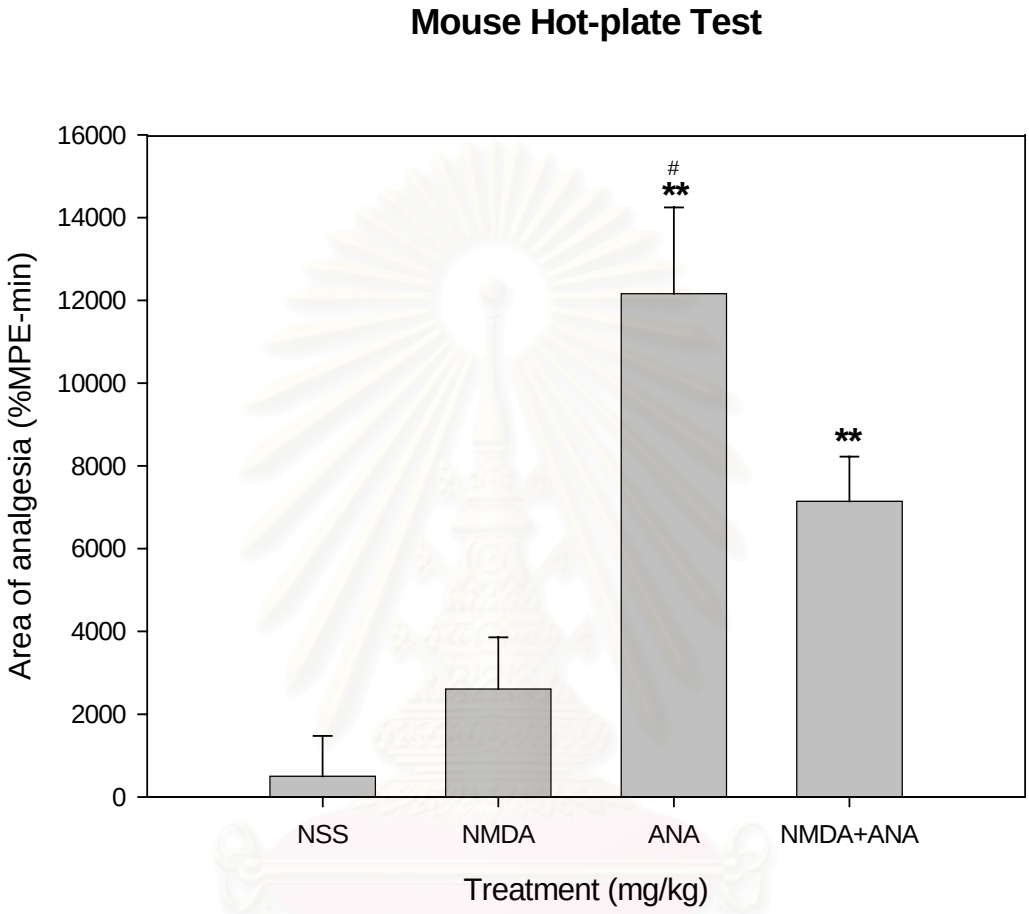


Figure 13 Area of analgesia (%MPE-min) from 0-240 minutes after intraperitoneal administration of 0.9% normal saline solution (NSS), ANA (500 mg/kg), NMDA 0.38 mg/kg, and NMDA+ANA (0.38/500 mg/kg). N=10 for all groups. ** $p<0.01$ significantly different compared to NSS, # $p < 0.05$ significantly different compared to NMDA+ANA (0.38/500 mg/kg).

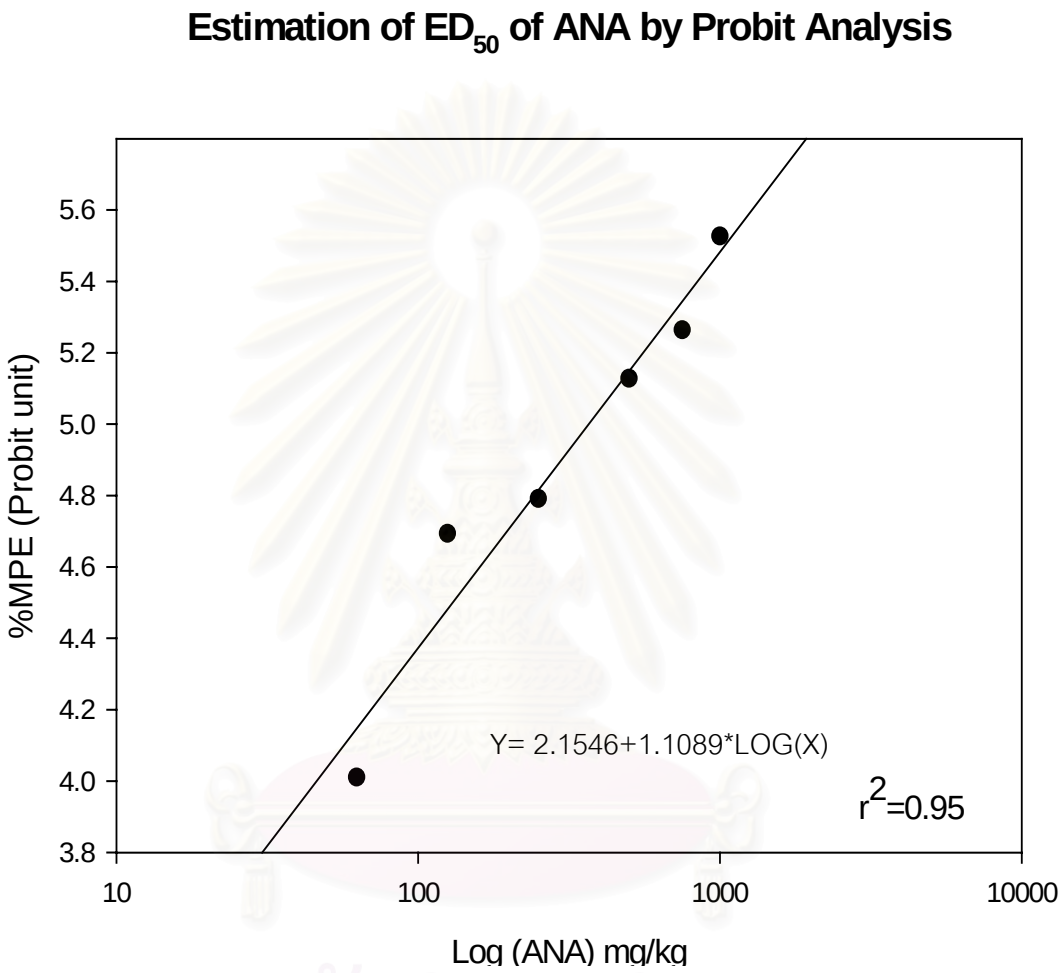


Figure 14 Linear regression of %MPE (Probit unit) at 45 minutes after intraperitoneal administration of various doses of ANA (62.5-1,000 mg/kg) using hot-plate test. N=10 for all groups. The ED_{50} was calculated from the log dose probit line as Y= 2.1546+1.1089*LOG(X) r^2= 0.95 and equal to 363.08 (59.23-2225.68) mg/kg.

MOUSE TAIL-FLICK TEST

To demonstrate the validity of the mouse tail-flick analgesic testing following i.p. drug administration, mice received morphine sulphate (MO; 10 mg/kg) i.p. and were tested during the subsequent 240 min period. As expected MO significantly ($p<0.01$) increased tail-flick latency producing an area of analgesia of 10701.61±1377.8 %MPE-min compared with that of NSS (-1,718.18±786.85 %MPE-min; Figure 15). The i.p. administration of ASA (150 mg/kg) also influenced the tail-flick latency and area of analgesia when compared to NSS ($p<0.01$; Figure 16).

Studies then utilized the mouse tail-flick method to examine the efficacy of ANA in producing analgesia. Mice were injected i.p. NSS or various doses of ANA (62.5, 125, 250 , 500, 750, 1,000 mg/kg). All doses of ANA significantly ($p < 0.05$, $p < 0.01$, $p < 0.01$, $p < 0.01$, $p < 0.01$, $p < 0.01$, respectively) increased tail-flick latency when compared to NSS. Additionally, all doses of ANA also significantly ($p<0.05$, $p<0.05$, $p<0.01$, $p<0.01$, $p<0.01$, respectively) increased tail-flick latency when compared to the lowest dose of ANA used (Figure 17). MO showed the highest analgesic response compared to all test groups. ANA dose 125 mg/kg produced analgesic response similar to ASA (Figure 18).

When the log dose of ANA was plotted versus the area of analgesia a significant linear correlation was observed. All six doses of ANA (62.5, 125, 250, 500, 750, 1,000 mg/kg) were plotted a significant linear correlation coefficient (r^2) equal to 0.98 was observed (Fig.19).

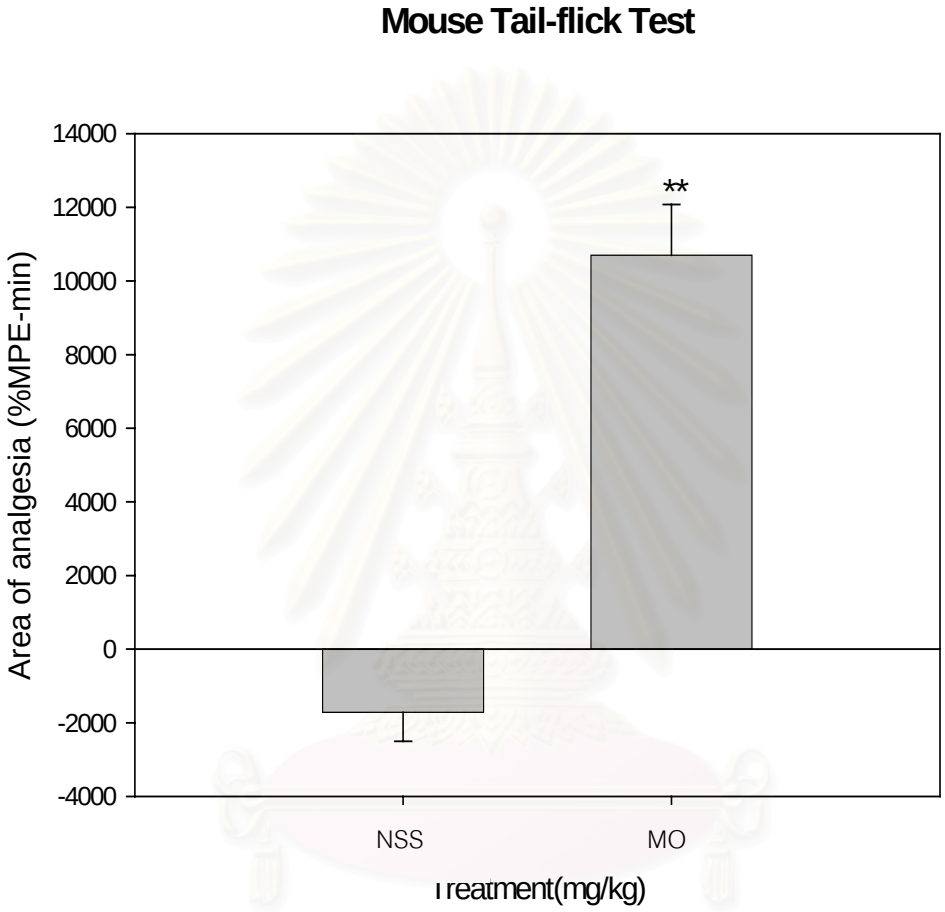


Figure 15 Area of analgesia (%MPE-min) from 0-240 minutes after intraperitoneal administration of 0.9% normal saline solution (NSS) and morphine sulphate (MO; 10 mg/kg). N=10 for all groups. ** $p < 0.01$ significantly different compared to NSS.

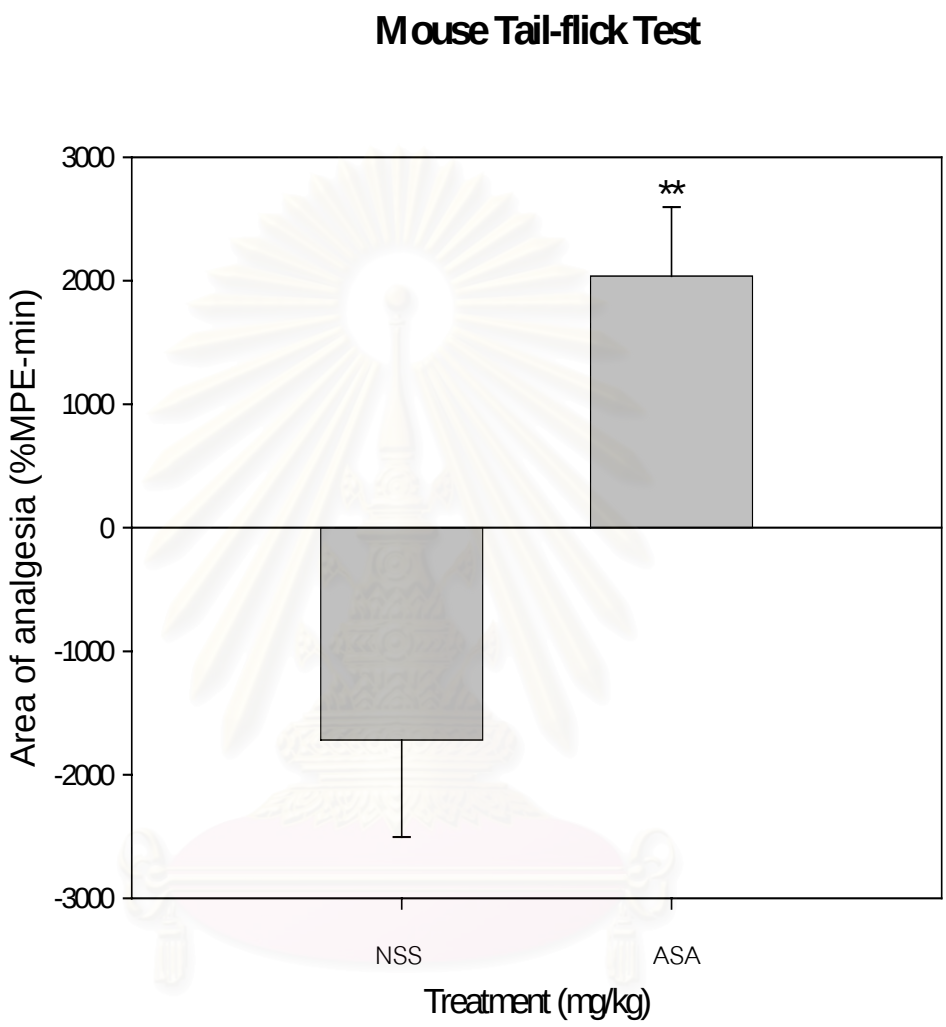


Figure 16 Area of analgesia (%MPE-min) from 0-240 minutes after intraperitoneal administration of 0.9% normal saline solution (NSS) and acetylsalicylic acid (ASA; 150 mg/kg). N=10 for all groups. ** $p < 0.01$ significantly different compared to NSS.

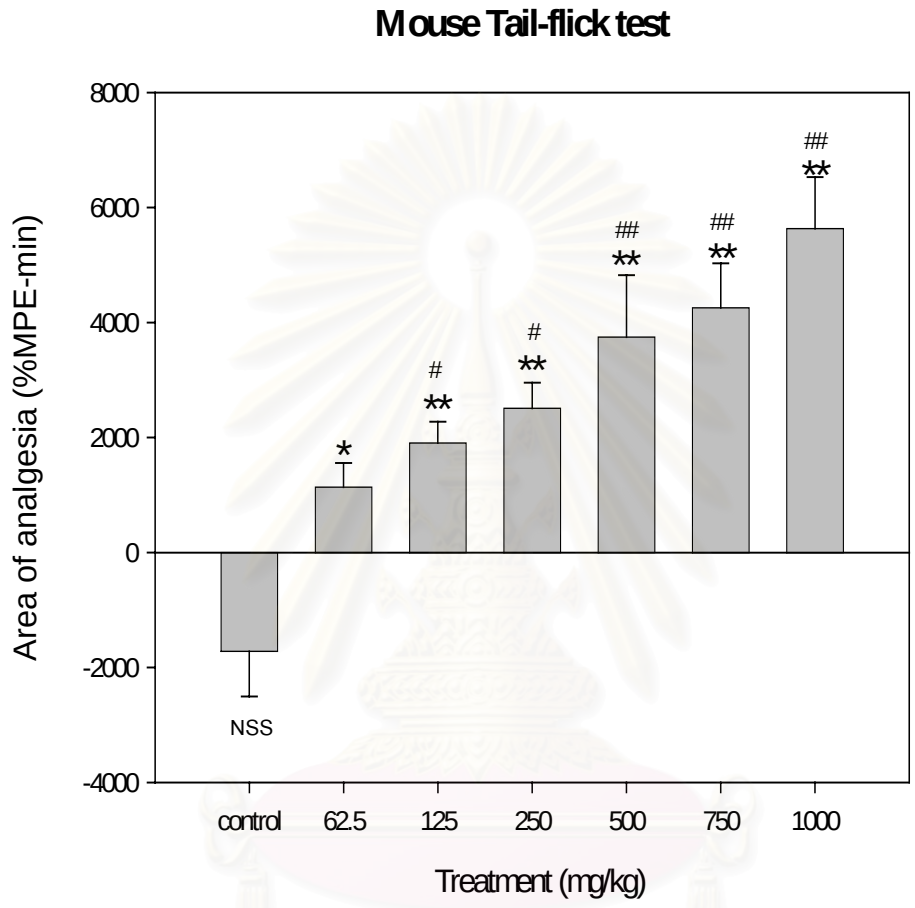


Figure 17 Area of analgesia (%MPE-min) from 0-240 minutes after intraperitoneal administration of 0.9% normal saline solution (NSS) and various doses of *Anacardium occidentale* leaves extract (ANA; 62.5-1,000 mg/kg). N=10 for all groups. *$p < 0.05$, **$p<0.01$ significantly different compared to NSS, # $p< 0.05$, ## $p< 0.01$ significantly different compared to ANA 62.5 mg/kg.

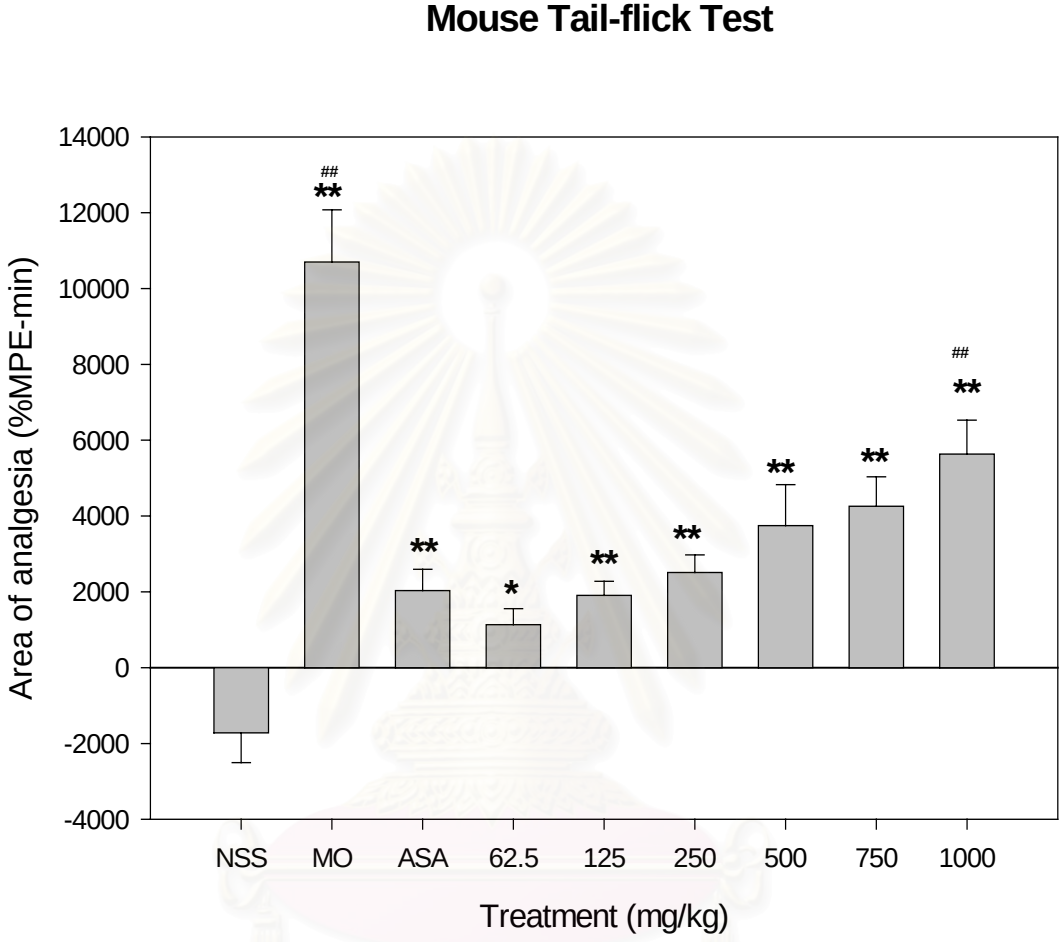


Figure 18 Area of analgesia (%MPE-min) from 0-240 minutes after intraperitoneal administration of 0.9% normal saline solution (NSS), MO (10 mg/kg), ASA (150 mg/kg), and various doses of ANA (62.5-1,000 mg/kg). N=10 for all groups. *$p < 0.05$, ** $p < 0.01$ significantly different compared to NSS, # $p < 0.05$, ## $p < 0.01$ significantly different compared to ANA 62.5 mg/kg.

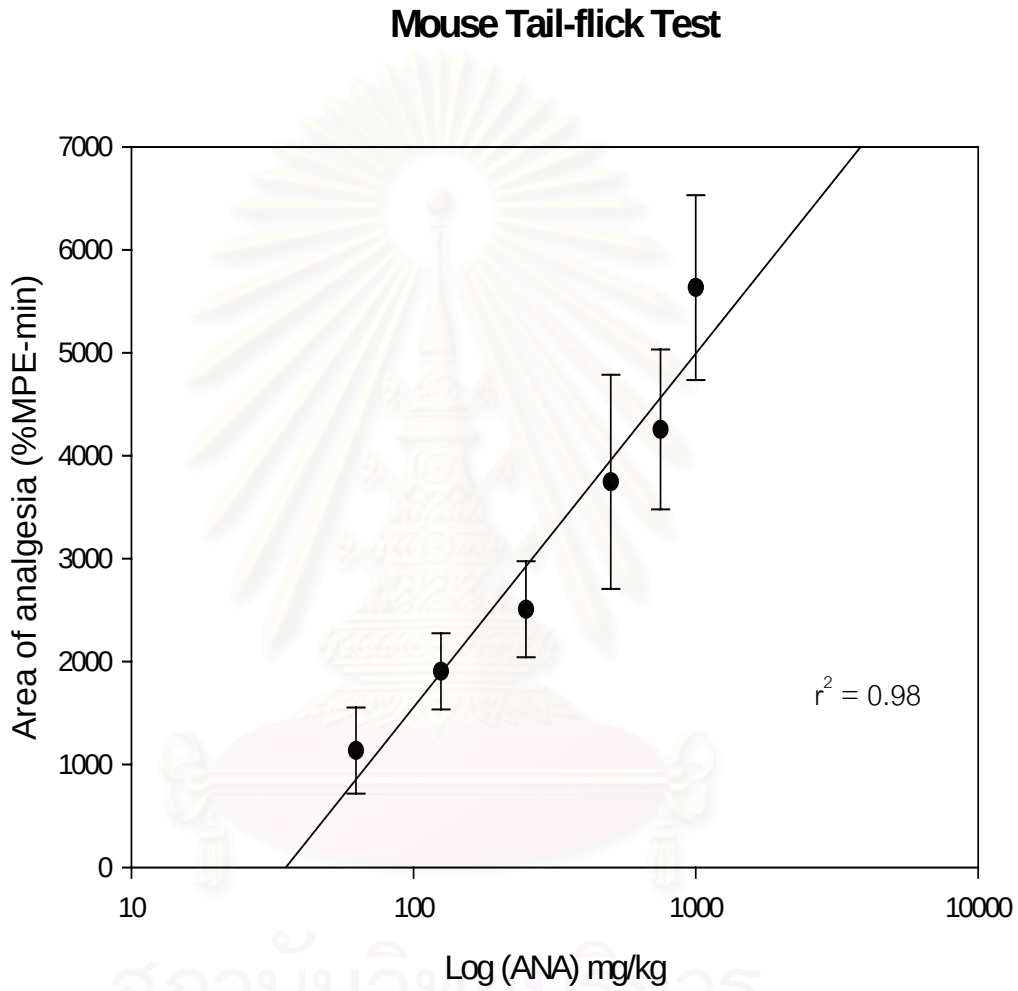


Figure 19 Linear regression of area of analgesia (%MPE-min) from 0-240 minutes after intraperitoneal administration of *Anacardium occidentale* leaves extract (ANA; 62.5-1,000 mg/kg). N = 10 for all groups. The regression equation was $Y = 879.21X + 120.37$, $r^2 = 0.98$

RAT PAW PRESSURE TEST

The studies were then conducted utilizing the Randall Selitto paw pressure technique. Initially, rats received i.p. NSS (1 ml/kg) or MO (10 mg/kg) and were tested during the subsequent 240 min period. MO significantly ($p<0.01$) increased the area of analgesia compared to vehicle group (Figure 20). The i.p. administration of ASA (150 mg/kg) also increased the pressure threshold and area of analgesia when compared to NSS ($p<0.01$; Figure 21).

In order to examine the efficacy of ANA in producing analgesia when the animals were stimulated by mechanical stimuli. Rats were injected i.p. NSS or various doses of ANA (62.5, 125, 250, 500, 750, 1,000 mg/kg). ANA doses of 250 mg/kg or higher significantly ($p< 0.05$, $p< 0.05$, $p< 0.01$, $p< 0.01$, respectively) increased the area of analgesia when compared to NSS. Additionally, ANA doses of 750 mg/kg and the highest dose also significantly ($p<0.01$, $p<0.01$, respectively) increased the area of analgesia when compared to the lowest dose of ANA used (Figure 22). ANA dose 500 mg/kg appears to produce the area of analgesia similar to MO and ASA (Fig.23).

When the log of the ANA dose was plotted versus the area of analgesia a significant linear correlation was observed. When all six doses of ANA (62.5, 125, 250, 500, 750, 1000 mg/kg) were plotted a linear correlation coefficient (r^2) equal to 0.84 was observed, while the plotting of only five doses (62.5, 125, 250, 500, 750 mg/kg) revealed a linear correlation coefficient of 0.98 (Figure 24& 25).

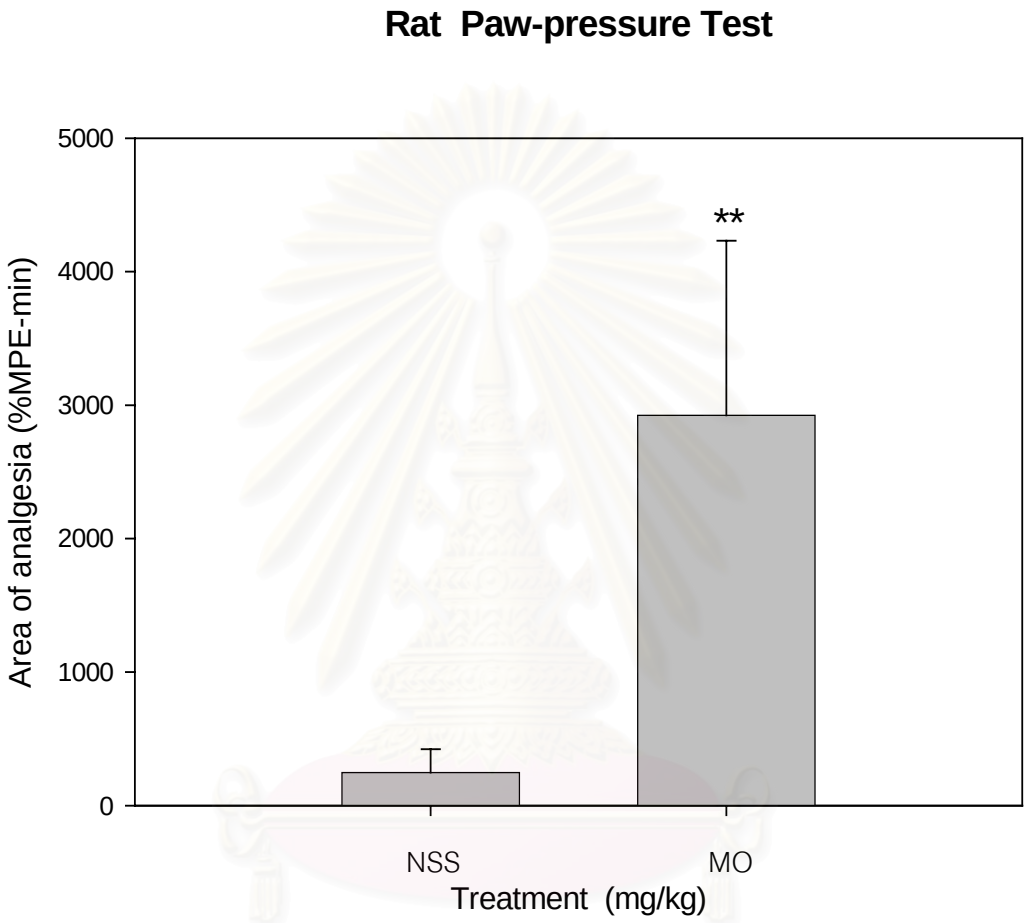


Figure 20 Area of analgesia (%MPE-min) from 0-240 minutes after intraperitoneal administration of 0.9% normal saline solution (NSS) and morphine sulphate (MO; 10 mg/kg). N=10 for all groups. ** $p < 0.01$ significantly different compared to NSS.

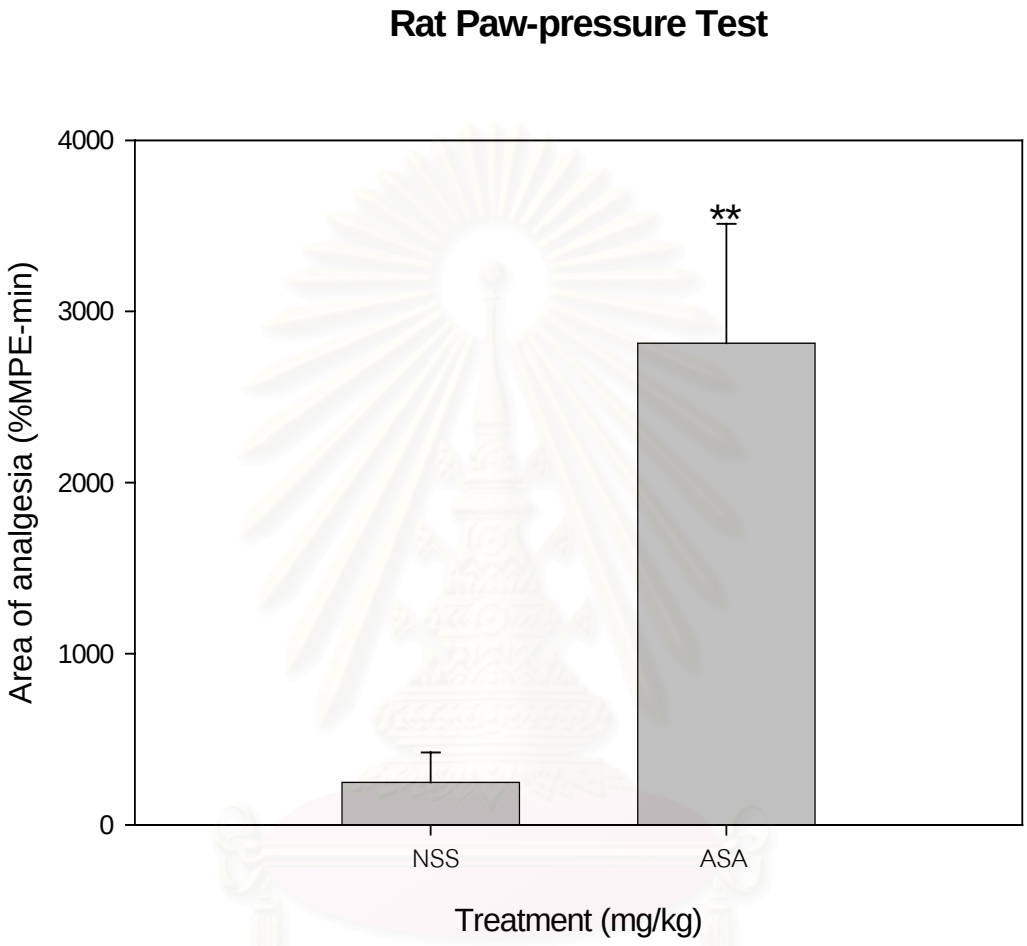


Figure 21 Area of analgesia (%MPE-min) from 0-240 minutes after intraperitoneal administration of 0.9% normal saline solution (NSS) and acetylsalicylic acid (ASA; 150 mg/kg). N=10 for all groups. ** $p<0.01$ significantly different compared to NSS.

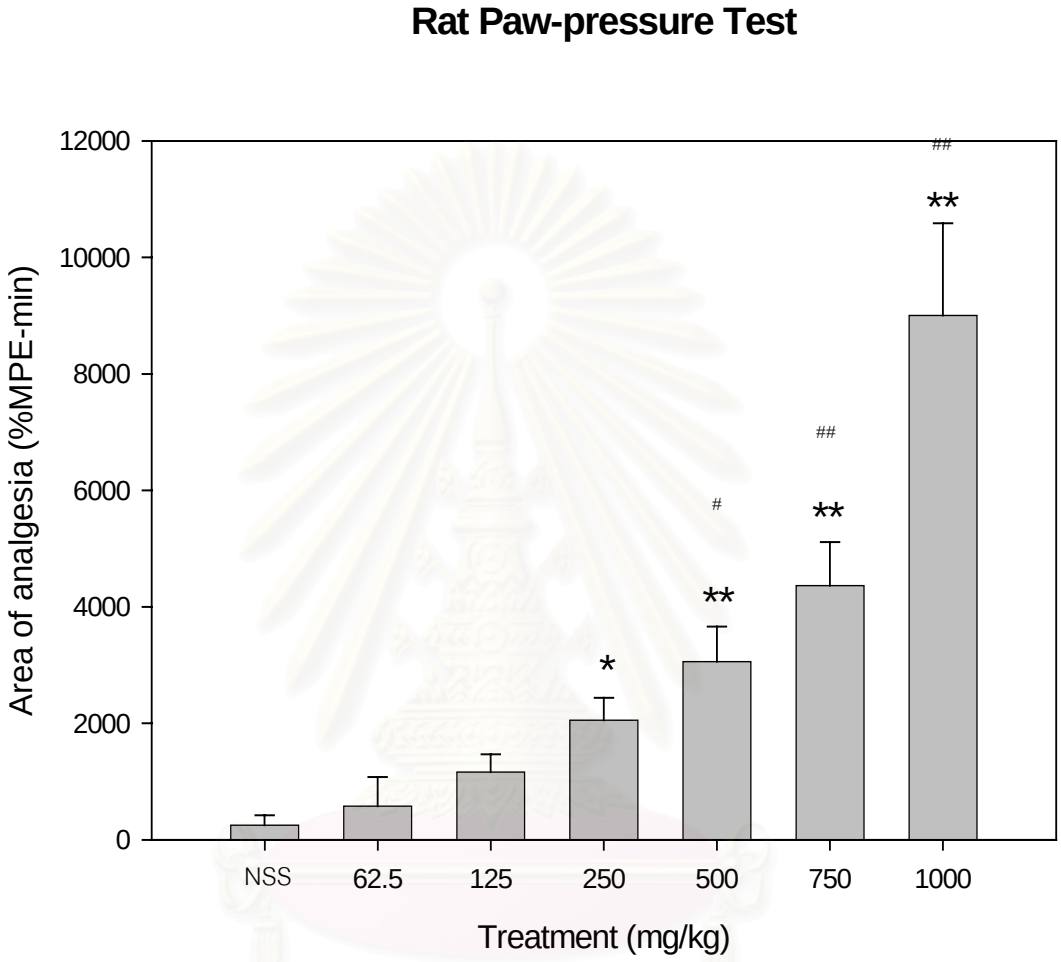


Figure 22 Area of analgesia (%MPE-min) from 0-240 minutes after intraperitoneal administration of 0.9% normal saline solution (NSS), and various doses of ANA (62.5-1,000 mg/kg). N=10 for all groups. * $p<0.05$, ** $p< 0.01$ significantly different compared to NSS, # $p< 0.05$, ## $p< 0.01$ significantly different compared to ANA 62.5 mg/kg.

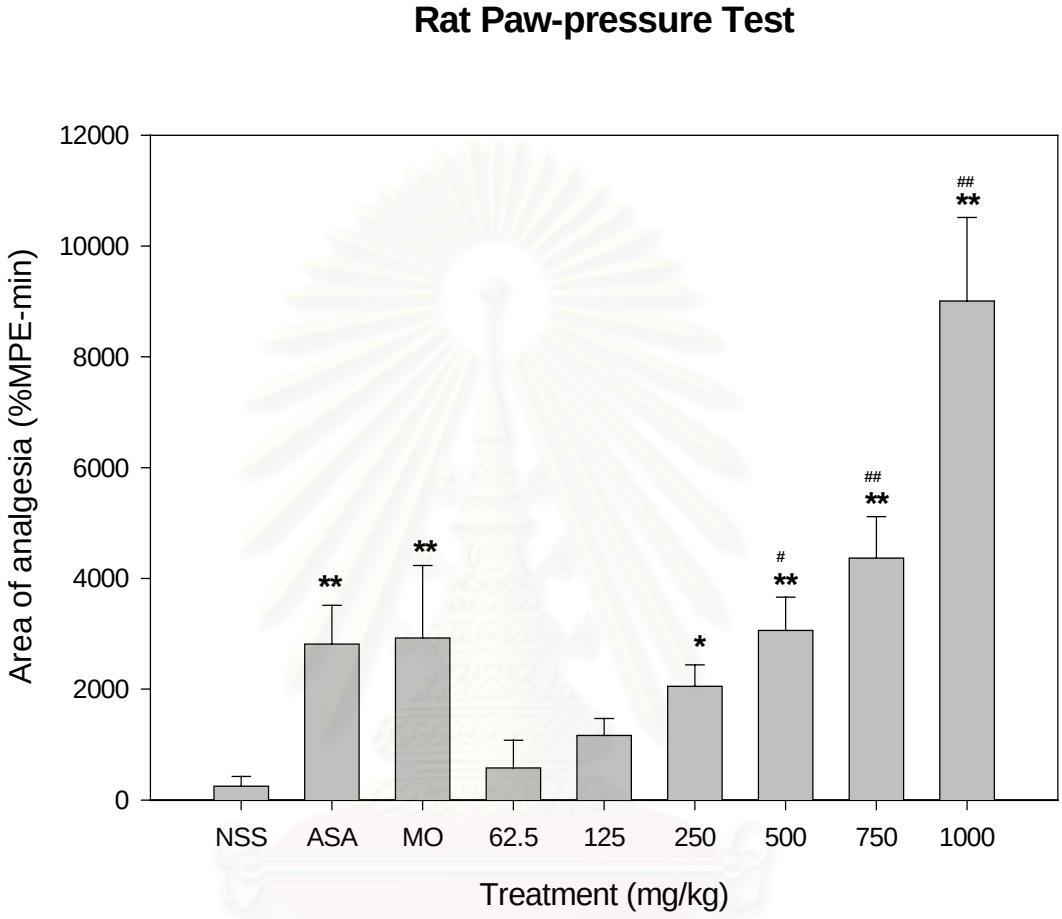


Figure 23 Area of analgesia (%MPE-min) from 0-240 minutes after intraperitoneal administration of 0.9% normal saline solution (NSS), morphine sulphate (MO; 10 mg/kg), acetylsalicylic acid (ASA; 150 mg/kg), and various doses of ANA (62.5-1,000 mg/kg). N=10 for all groups. * $p<0.05$, ** $p< 0.01$ significantly different compared to NSS, # $p< 0.05$, ## $p< 0.01$ significantly different compared to ANA 62.5 mg/kg.

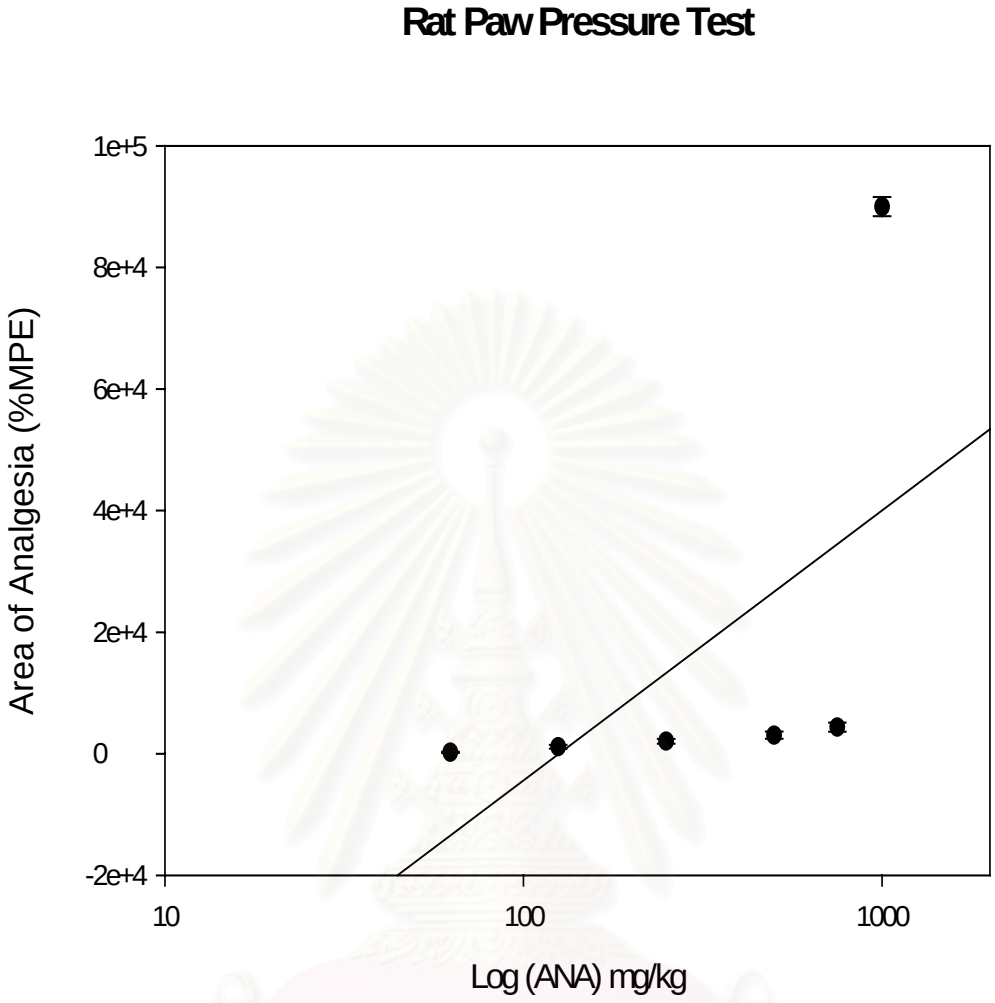


Figure 24 Linear regression of area of analgesia (%MPE-min) from 0-240 minutes after intraperitoneal administration of various doses of *Anacardium occidentale* leaves extract (ANA; 62.5-1,000 mg/kg). N = 10 for all groups. The regression equation was $Y = 1506.9X - 1903.6$, $r^2 = 0.84$

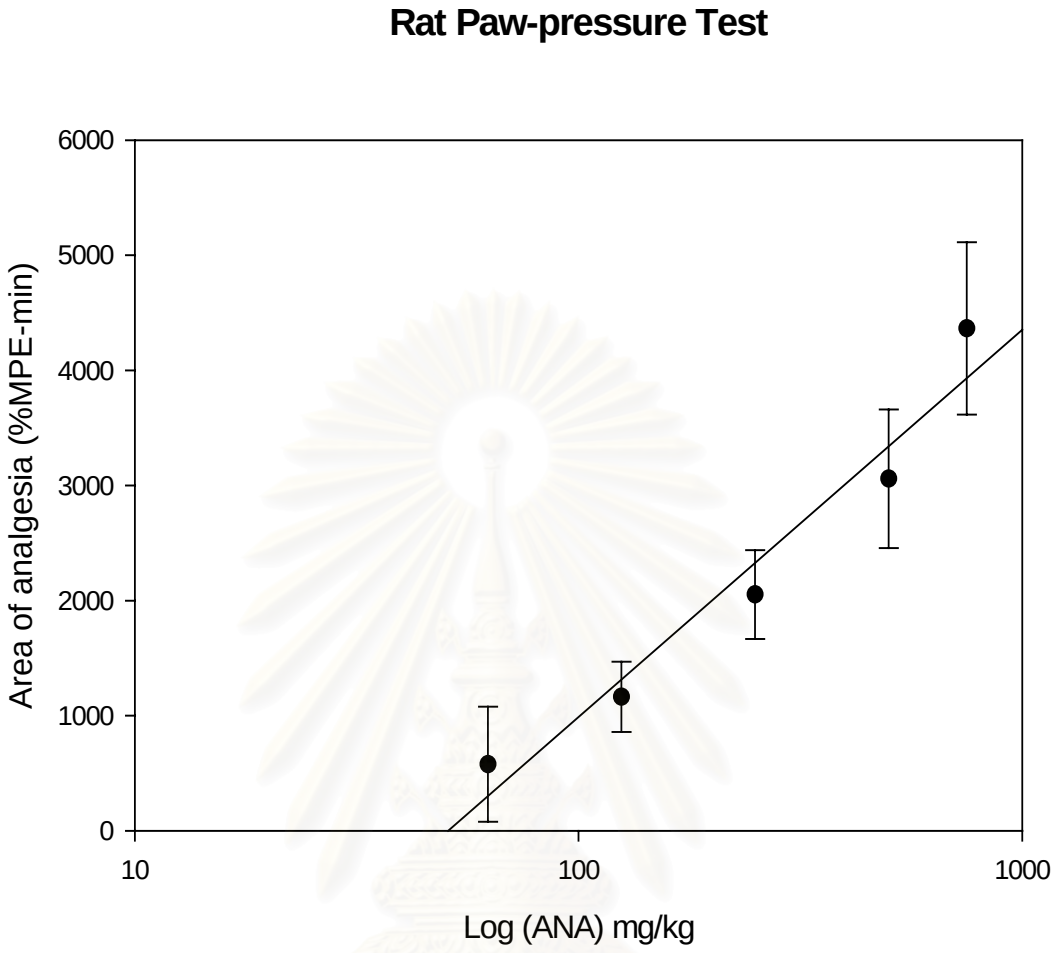


Figure 25 Linear regression of area of analgesia (%MPE-min) from 0-240 minutes after intraperitoneal administration of various doses of *Anacardium occidentale* leaves extract (ANA; 62.5-750 mg/kg). N = 10 for all groups. The regression equation was $Y = 946.74X - 596.49$, $r^2 = 0.98$

INFLAMED PAW-PRESSURE TEST

To determine the antihyperalgesic effect of ANA in carrageenan induced paw inflammation, the Randall Selitto paw pressure technique was used. Two hours after the carrageenan injection into the plantar surface of rat's right hind paw, the right hind paw was red and swollen. The contra-lateral, left hind paw appeared unaffected. In control group rats (receiving a systemic injection of 0.9%NSS i.p.), withdrawal threshold after mechanical stimulation of inflamed paw (76 $\pm$ 5.57) were significantly shorter than the non-inflamed paw (96.25 $\pm$ 5.03), indicating a carrageenan induced hyperalgesia.

To demonstrate the validity of the paw-pressure technique following i.p. drug administration, rats received morphine (MO; 10 mg/kg) or indomethacin (IND; 5 mg/kg) i.p. 30 min prior administering carrageenan, two hours later, rats were tested for mechanical hyperalgesia. MO significantly (p<0.01) increased pressure threshold compared to NSS (Figure 26). The i.p. administration of IND (5 mg/kg) also increased pressure threshold when compared to NSS (p<0.01; Figure 27). r^2=0.98

To evaluate the antihyperalgesic effect of ANA, rats were received various doses of ANA (62.5, 125, 250, 500, 1,000 mg/kg) i.p. 30 min before carrageenan administration. ANA doses of 250, 500, and 1000 significantly ($p < 0.05$, $p < 0.01$, p <0.01, respectively) increased pressure threshold when compared to NSS. The highest dose of ANA also significantly (p<0.01) increased pressure threshold when compared to the ANA 62.5 mg/kg group (Figure 28). Additionally, ANA doses of 250 mg/kg and higher significantly (p<0.05, p<0.01, p<0.01, respectively) exerted analgesic effect on both inflamed and intact paws when compared to NSS treated group. ANA dose 125 mg/kg increased withdrawal threshold similar to MO and ASA (Figure 29).

When the log dose of ANA was plotted versus the area of analgesia a significant linear correlation was observed. All five doses of ANA (62.5, 125, 250, 500, 750, 1,000 mg/kg) were plotted a significant linear correlation coefficient (r^2) equal to 0.98 was observed (Fig.30).

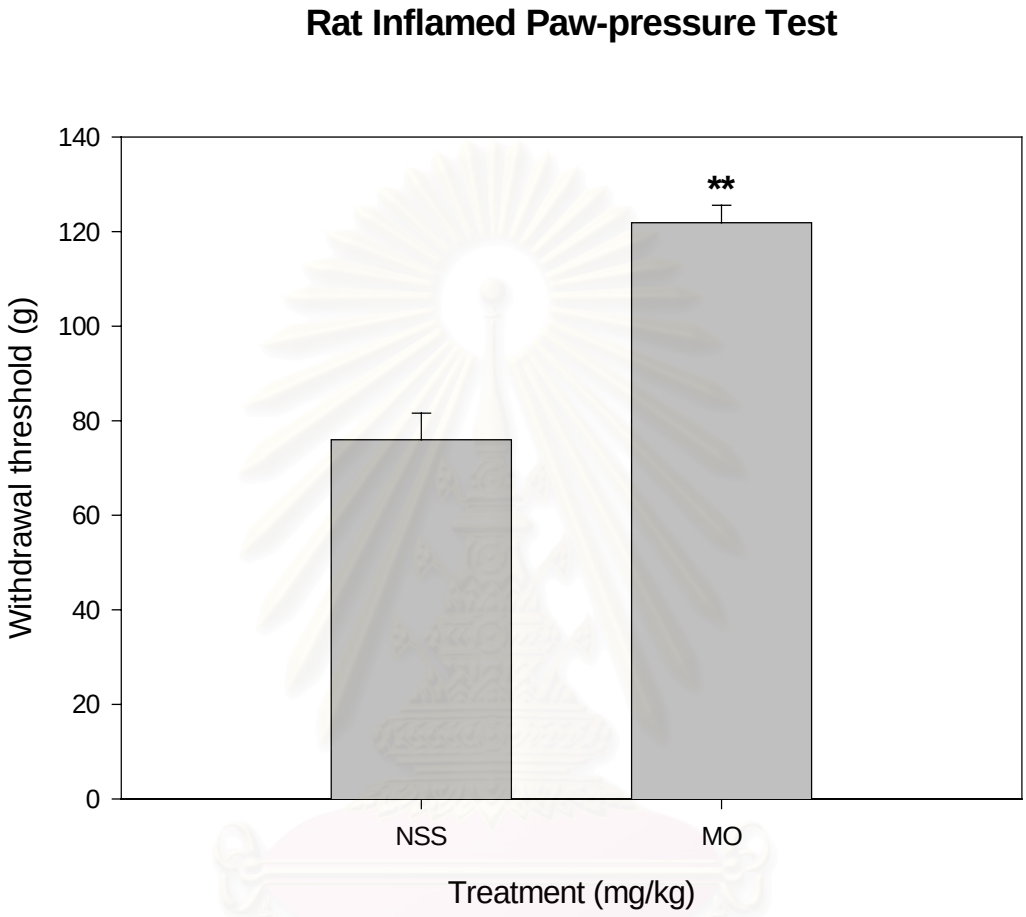


Figure 26 Withdrawal threshold pressure from 0-240 minutes after intraperitoneal administration of 0.9% normal saline solution (NSS; N=6) and morphine sulphate (MO; 10 mg/kg, N=8) ** $p < 0.01$ significantly different compared to NS S treated group.

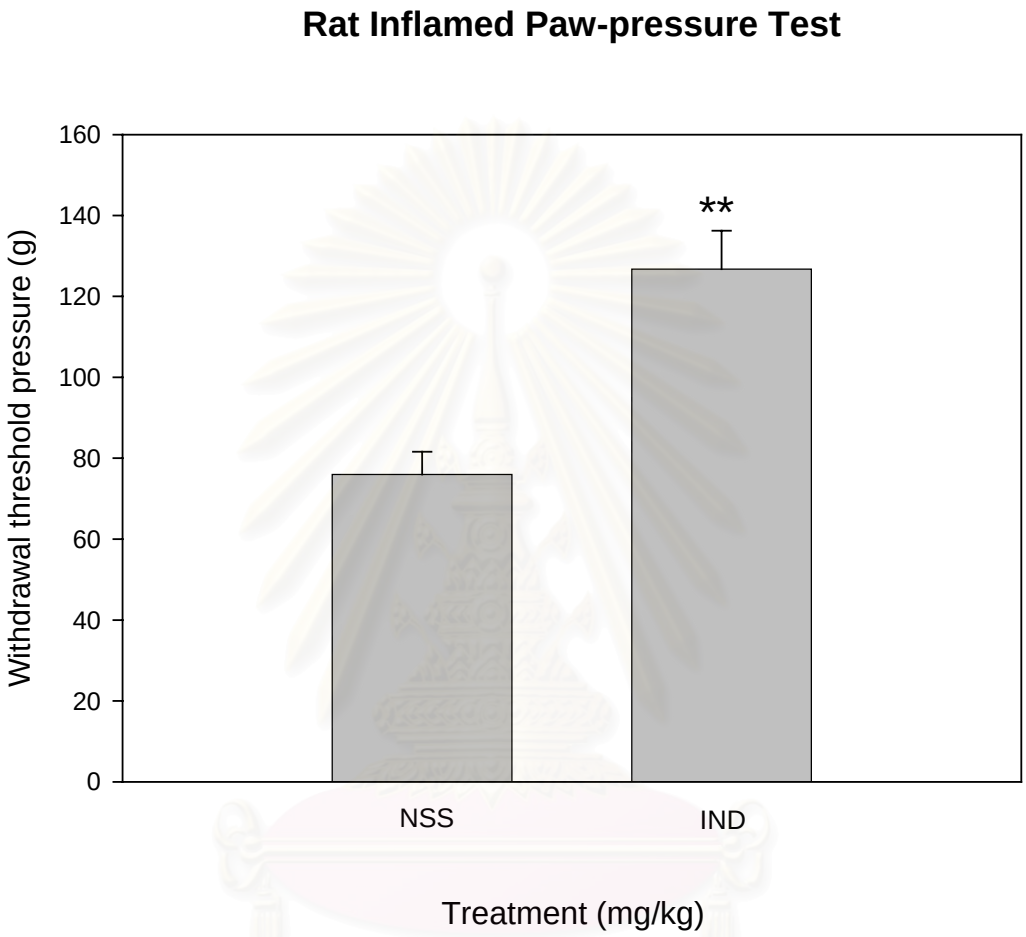


Figure 27 Withdrawal threshold pressure from 0-240 minutes after intraperitoneal administration of 0.9% normal saline solution (NSS; N=6) and indomethacin (IND; 5 mg/kg, N=8). ** $p < 0.01$ significantly different compared to NSS.

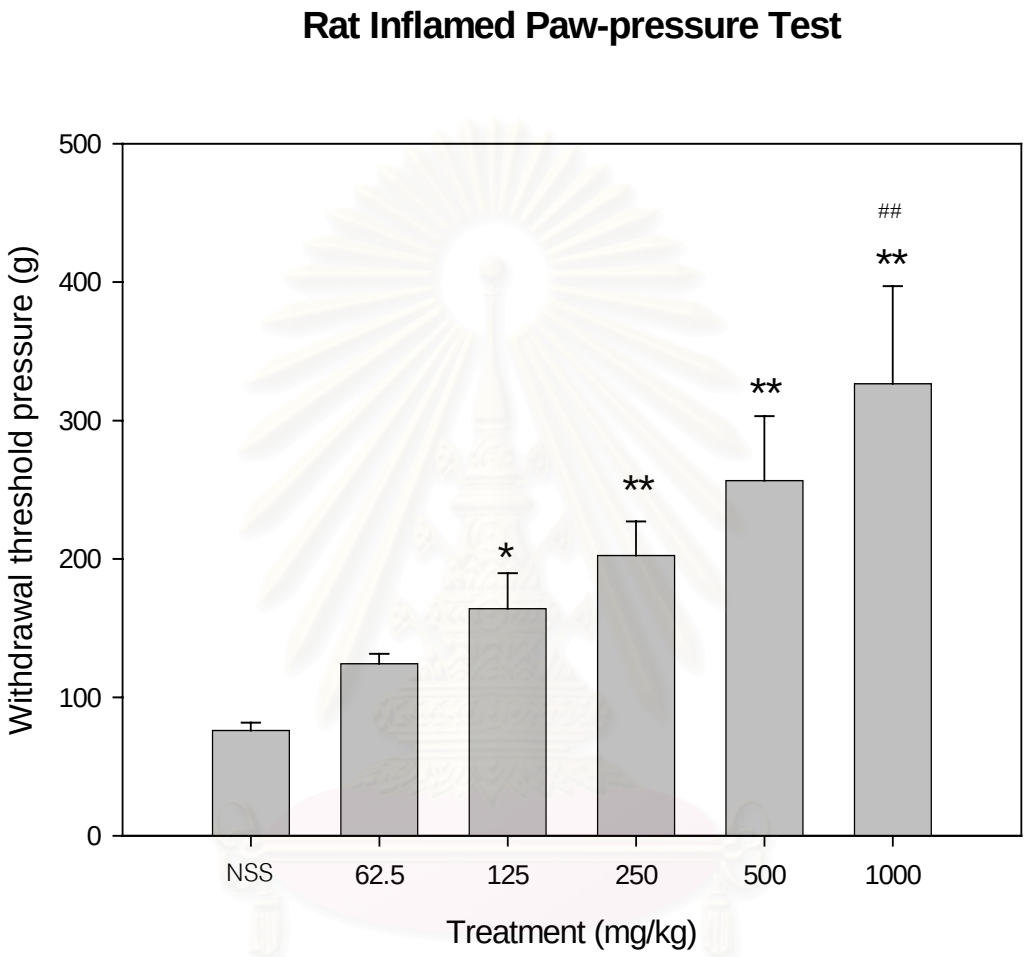


Figure 28 Withdrawal threshold pressure from 0-240 minutes after intraperitoneal administration of 0.9% normal saline solution (NSS; N=6) and various doses of *Anacardium occidentale* leave extract (ANA; 62.5-1,000 mg/kg, N=8). * $p < 0.05$, ** $p < 0.01$ significantly different compared to NSS treated group, ## p <0.01 significantly different compared to ANA 62.5 mg/kg group.

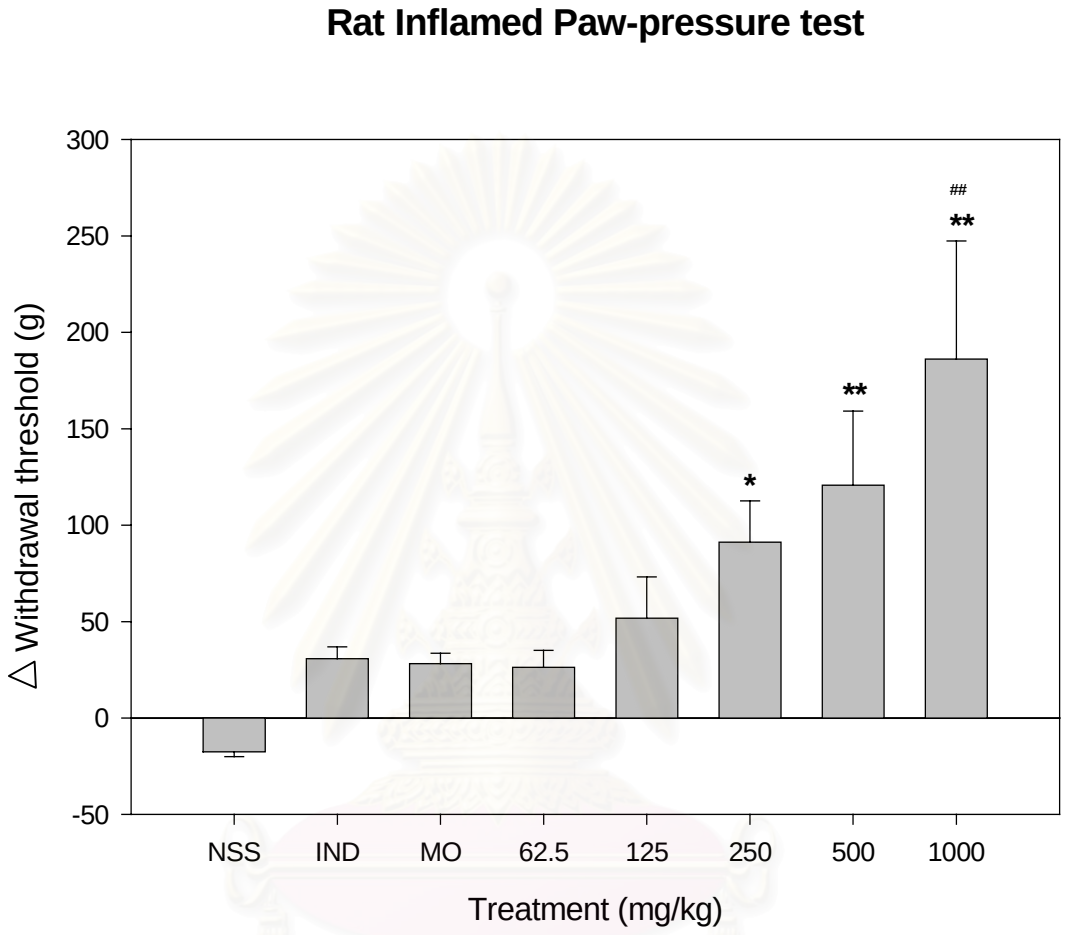


Figure 29 △ Withdrawal threshold pressure from 0-240 minutes after intraperitoneal administration of 0.9% normal saline solution (NSS; N=6), indomethacin (IND; 5 mg/kg, N=8), morphine sulphate (MO; 10 mg/kg, N=8), and various doses of *Anacardium occidentale* leave extract (ANA; 62.5-1,000 mg/kg, N=8). * $p < 0.05$, ** $p < 0.01$ significantly different compared to NSS treated group, ## p<0.01 significantly different compared to ANA 62.5 mg/kg

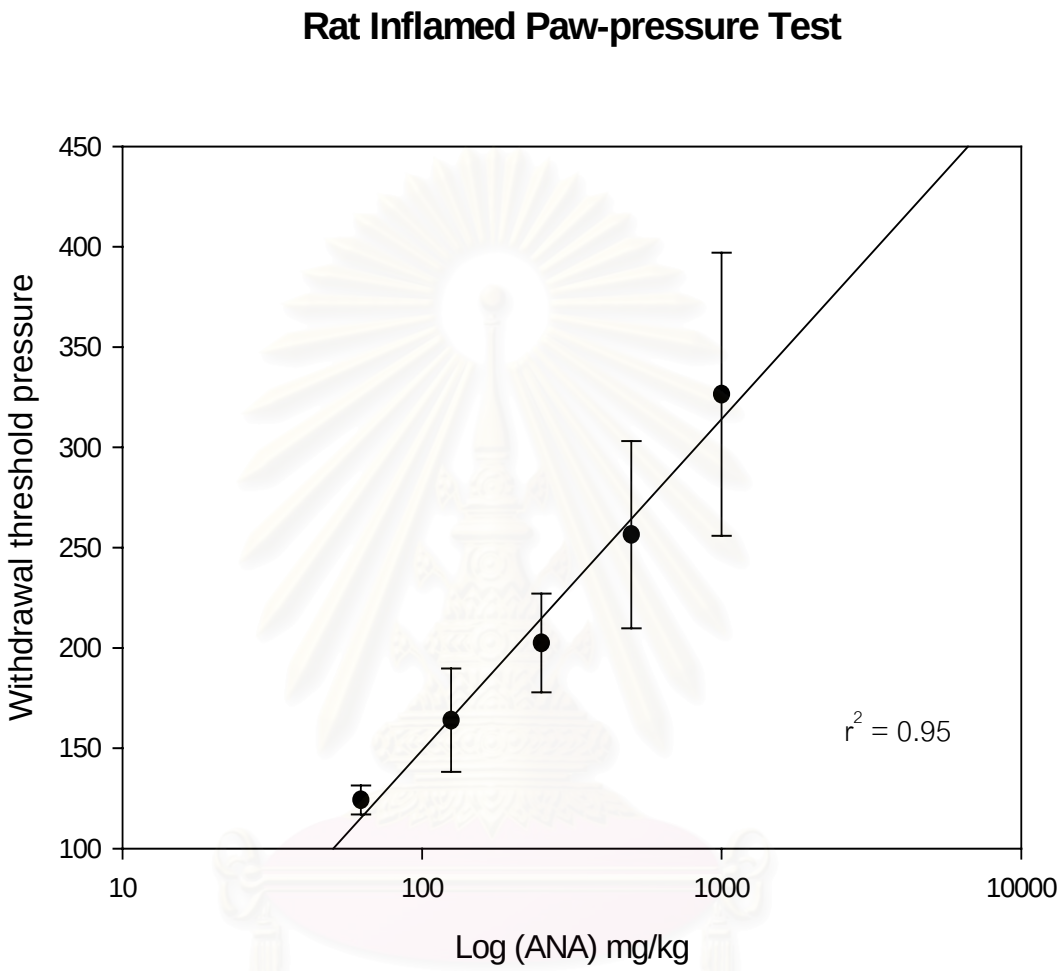


Figure 30 Linear regression of paw withdrawal threshold from 0-240 minutes after intraperitoneal administration of various doses of *Anacardium occidentale* leaves extract (ANA; 62.5-1,000 mg/kg). N = 8 for all groups. The regression equation was $Y = 0.2023X+136.38$, $r^2 = 0.95$

ROTA-ROD TEST

In order to measure the effect of ANA on motor performance, mice were then treated i.p. with various doses of ANA (125, 250, 500 mg/kg) and tested on the rota-rod. The rotarod performance of mice was observed at 30, 60, 120, and 240 min after ANA injection. Data showed that all doses of ANA tested did not significantly produce motor impairment (Figure 31). TD_{50} was calculated from log dose probit line and was equal to 794.33 mg/kg (Figure 32).

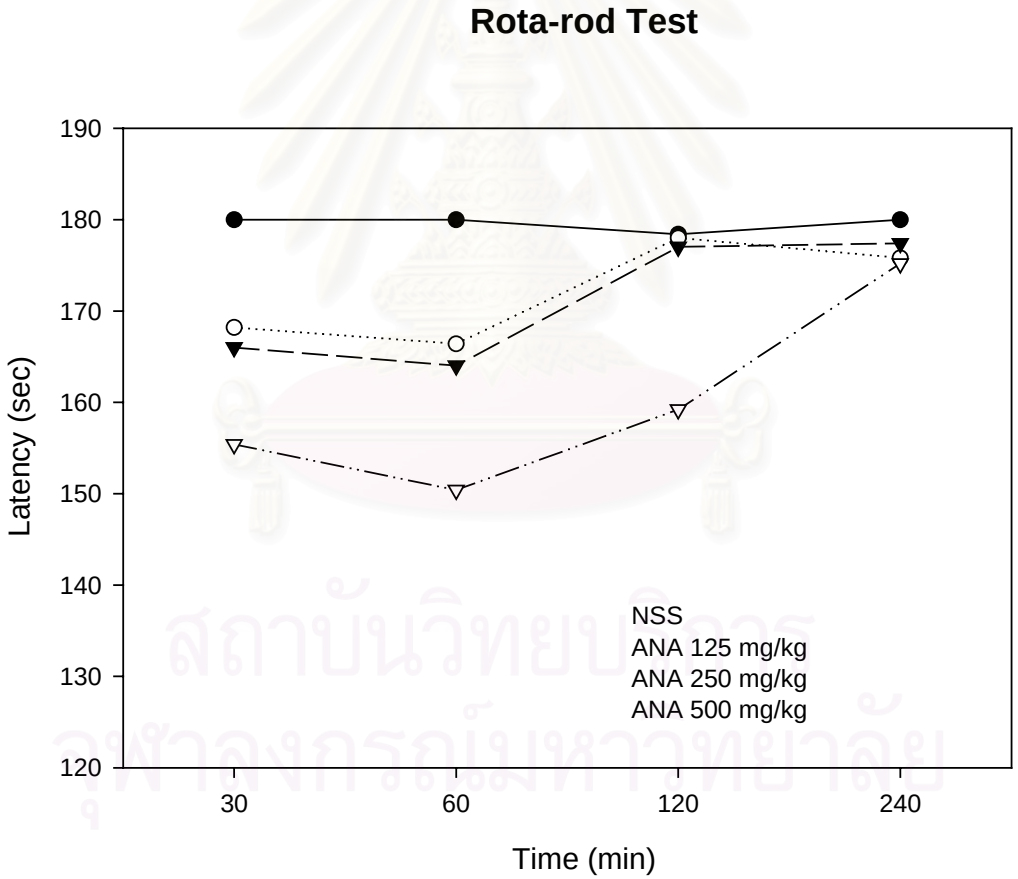


Figure 31 Rota-rod latency of various doses of *Anacardium occidentale* leaves extract (ANA; 250-500 mg/kg) on rota-rod test at 30, 60, 120 and 240 min. after intraperitoneal administration.

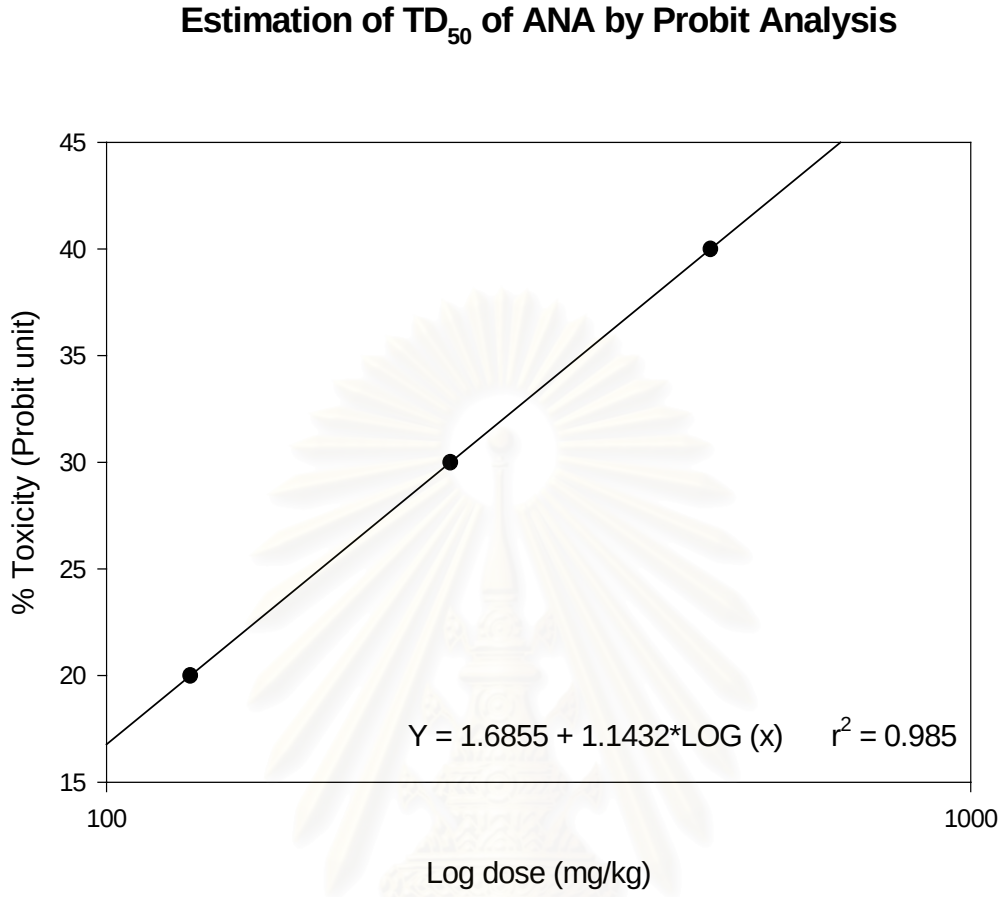


Figure 32 Linear regression of %toxicity (Probit unit) 0-240 min after intraperitoneal administration of various doses of ANA (125, 250, and 500 mg/kg). N=10 for all groups. TD_{50} = 794.33 (136.25-4630.94) mg/kg. The TD_{50} was calculated from the log dose probit line as Y = 1.6855 + 1.1432 * LOG(x) r^2= 0.985

ACUTE TOXICITY TEST AND LD_{50} DETERMINATION

The LD_{50} was estimated based on 50% death within 72 h following intraperitoneally injection of *Anacardium occidentale* leaves extract (ANA). ANA doses of 62.5,125, and 250 mg/kg did not induce any death in this experiment. In contrast, ANA doses of 500, 750 and 1,000 mg/kg produced lethality rate of 26.67%, 66.67% and 86.67%, respectively in animals tested. Using Litchfield and Wilcoxon method, the LD_{50} was estimated to be 598.69 mg/kg.

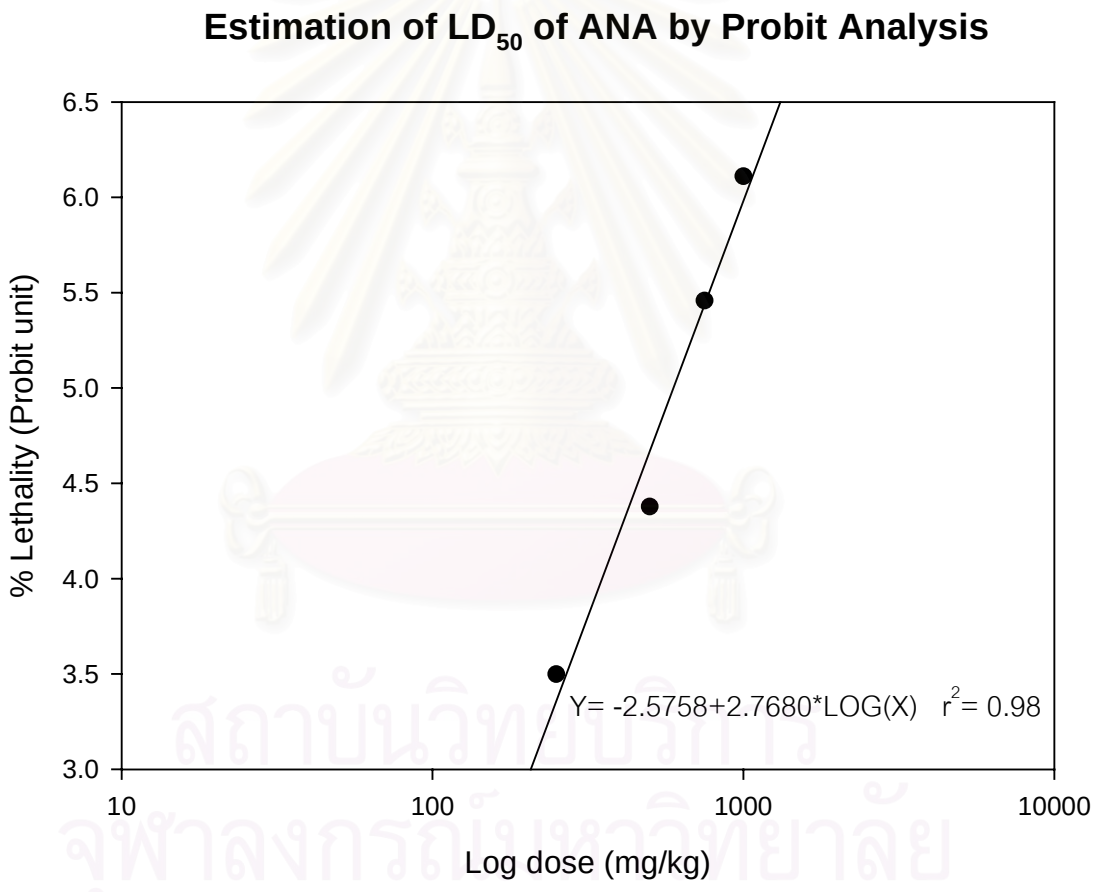


Figure 33 Linear regression of %lethality (Probit unit) after intraperitoneal administration of various doses of ANA (62.5-1,000 mg/kg) within 72 h. N=10 for all groups. LD_{50} = 598.69 (284.87-1220.66) mg/kg. The LD_{50} was calculated from the log dose probit line as Y= -2.5758+2.7680*LOG(X) r^2= 0.98.

CHAPTER V

DISCUSSION & CONCLUSION

These studies have demonstrated the activity of the *Anacardium occidentale* leaves extracts (ANA) to generate significant analgesia in different animal models. Initial attempts to investigate the analgesic effect of ANA utilized the standard mice hot-plate technique. ANA doses of 125-1,000 mg/kg demonstrated a dose-response relationship with the mouse hot-plate technique. The lowest dose of ANA (62.5 mg/kg) had no analgesic effect in this model (Figure 7). The linear regression equation for all doses of ANA was [Response = 2158.5dose + 1612.6], r^2= 0.87and [Response = 994.37dose + 7139], r^2= 0.98 when excluding the highest and the lowest doses of ANA (Figure 9&10). The analgesic action of ANA was observed during 240 min period (Figure 11). Morphine (MO) as a reference standard had shown potent analgesic effect but acetylsalicylic acid had little influence on the response in this animal model. All doses of ANA except 62.5 mg/kg appeared to produce higher analgesic response compared to ASA and the highest dose of ANA produced analgesic response similar to MO (Figure 8). The ED_{50} was equal to 363.08 mg/kg (Figure 14).

NAL was utilized to investigate the involvement of opioid receptor in the analgesic effects of ANA. The results showed the involvement of opioid receptor in analgesia produced by ANA (Figure 12). Since NMDA coadministration appeared to attenuate the analgesic response of ANA, suggested also an involvement of NMDA receptor in ANA analgesia (Figure 13).

Studies were then undertaken to investigate the effectiveness of ANA utilizing the mouse tail-flick technique. MO administered i.p. produced significant analgesic response as expected (Figure 15). ASA also produced analgesia but at a lesser extent compared to MO in this animal model (Figure 16). All doses of ANA administered i.p. produced a dose related analgesic response and ANA dose 125 mg/kg appeared to

produce analgesic effect similar to ASA (Figure 18). The linear regression equation for all doses of ANA was [Response = 879.21dose + 120.37], r^2= 0.898 (Figure 19).

The Randall Selitto paw-pressure test was chosen to measure ANA effect against mechanical stimuli. Intraperitoneal administration of MOR and ASA at dose tested produce similar analgesic response compared to NSS treated controls utilizing this method (Figure 20&21). Similar to mouse hot-plate and tail-flick tests, ANA demonstrated a dose-response relationship with the rat paw-pressure technique (Figure 22). The linear regression equation of all doses of ANA was [Response = 1506.9dose -1903.6], r^2= 0.84 and [Response = 946.74dose -596.49], r^2= 0.98 when excluding the highest dose of ANA (Figure 24&25). ANA dose 500 mg/kg appeared to produce analgesic response similar to MO and ASA (Figure 23).

In an attempt to confirm the analgesic response of ANA, we compared withdrawal threshold observed from an intact paw with and an inflamed paw. The inflammation was induced by a subcutaneous injection of carrageenan into the subplantar region of rat's hind paw. Intraperitoneal administration of MO and IND at dose tested produce similar analgesic response compared to NSS treated controls utilizing this method (Figure 26&27). ANA doses of 250 mg/kg and higher inhibited carrageenan-induced hyperalgesia compared to NSS treated controls. ANA dose 125 mg/kg appeared to produce analgesic effect similar to MO and IND (Figure 28&29). Similar to the previous studies, ANA demonstrated a dose-response relationship with the rat inflamed paw-pressure technique (Figure 28&29). The linear regression equation of all doses of ANA was [Response = 0.2023dose + 136.38], r^2= 0.95 (Figure 30).

ANA has demonstrated analgesic response in all testing models suggesting that ANA could produce analgesia via both spinal and supraspinal mechanisms. The tail-flick analgesia testing seemed to be the most sensitive test for evaluating analgesic effect of ANA as all doses of ANA had shown higher analgesic response compared to NSS treated group.

Preliminary results had shown that ANA dose 1,000 mg/kg administered orally produced no death while all animals received same dose of ANA i.p. died within 72 hours suggesting that ANA toxicity could be reduced by the first pass effect. The toxicity of ANA may due to cardol that was found most often in the nut shell and probably found also in the leaves of *Anacardium occidentale*.

In order to evaluate the possible non-specific muscle relaxant or sedative effects of ANA, mice were tested on the rota-rod. Animals were treated with various doses of ANA (125, 250, 500 mg/kg) i.p. 30 min before being tested. ANA at all doses tested failed to significantly alter the rota-rod performance compared to NSS treated controls. Therefore, the antinociception caused by ANA is probably unrelated with impairment of motor response, as at doses in which the extract produced pronounced antinociception it had no significant effect on the motor function of animals, as demonstrated by the complete lack of effect when the rota-rod apparatus was used for testing.

In conclusion, the current study has demonstrated that as reported for many naturally-occurring substances isolated from plants, the ethanol fraction obtained from *Anacardium occidentale* leaves exerts a pronounced antinociception when assessed in thermal and mechanical models of nociception in rodents, these effects being due, at least in part, to the presence of phenolic compounds. The precise mechanisms involved in their action are, at this moment, not completely understood; they are most likely involved with both opioid pathway and NMDA receptor complex.

FUTURE RESEARCH

In these studies there was evidence that the ethanol extract of *Anacardium occidentale* leaves was capable of significantly produced analgesic and antihyperalgesic response, most likely via an opioid and NMDA-mediated mechanism, and supports the potential use of the extract.

The future research could comprise of several objectives as listed below:

(1) To identify and remove possible toxic substances from the extract.

(2) To investigate the antipyretic effect of various doses of ANA.

(3) To investigate the anesthetic effect of various doses of ANA.

(4) To investigate the potential use of ANA in combination with other analgesic or nonsteroidal anti-inflammatory drugs.

(5) To investigate other routes of administration that might be more appropriate for the use of ANA and possibly enhance the analgesic effect of ANA.

(6) To better understand the mechanism of ANA that is involved in producing its analgesic and antihyperalgesic effects.

(7) To observe other opioid antagonist to better characterize the mechanism of ANA effect.

(8) To observe other NMDA receptor agonist to better characterize the mechanism of ANA effect.

(9) To test side effects and toxic effects of ANA at high doses.

These and other studies may provide important clues to help understand the mechanisms underlying the analgesic effect of ANA and further support the use of such compounds in a clinical setting.

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APPENDIX

Table 1: %MPE-Time in Mouse Hot-plate test 0-240 min of ANA dose 62.5-1,000 mg/kg i.p. data present as mean %MPE ± S.E.M. N=10 per group.

Dose ANA (mg/kg)	15 min	30min	45 min	60 min	90 min	120 min	240 min
62.5	14.34 ± 7.47	15.61±7.1	23.38 ± 7.67	16.12 ± 5.86	-3.2 ± 2.25	-2.15 ± 2.93	2.81 ± 4.32
125	23.28± 6.17	30.58 ± 10.47	37.52 ± 7	35.87 ± 5.79	37.58 ± 8.63	24.91 ± 4.17	24.61 ± 7.37
250	25.08 ± 3.4	31.04 ± 8.89	48.96 ± 10.26	45.5 ± 7.75	41.74 9.22	19.73 ± 6.6	27.57 ± 10.83
500	27.05 ± 5.24	40.91 ±7.24	55.12 ± 11.9	49.37 ± 9.66	50.04 ± 7.58	39.4 ± 8.79	17.65 ± 6.57
750	33.77 ±11.6	49 ± 11.65	60.44 ± 12.9	58.64 ± 11.83	34.99 ± 12.82	43.15 ± 13.74	43.87 ± 15.21
1000	50 ± 9.3	64.26 ± 8.62	70.12± 8.62	69.54 ± 10	61.92 ± 12.09	61.3 ± 14.13	58.19 ± 9.72

Table 2: %MPE-Time in Mouse Tail-flick test 0-240 min of ANA dose 62.5-1,000 mg/kg i.p. data present as mean %MPE ± S.E.M. N=10 per group

Dose ANA (mg/kg)	15 min	30min	45 min	60 min	90 min	120 min	240 min
62.5	0.58 ± 1.79	3.44±1.8	9.35±3.5	5.83±3.2	0.99±2.44	4.93±2.9	2.18±2.15
125	5.88±2.11	6.48±3.29	13.89±2.98	5.39±2.08	6.44±2.27	5.47±2.59	8.99±3.27
250	6.85±2.11	9.05±2.41	14.96±6.9	12.4±3.84	14.89±5.56	10.59±1.85	8.79±4.36
500	7.81±3.36	10.38±2.57	26.33±12.48	13.15±3.88	13.62±5.56	13.52±3.62	12.83±3.1
750	8.06±2.74	10.64±2.48	23.88±7.37	20.95±8.32	18.88±3.22	18.16±4.29	19.29±6.4
1000	11.39±3.67	19.55±4.72	32.63±9.86	24.3±3.4	27.43±7.89	23.38±3.88	24.91±5.59

Table 3: %MPE-Time in Rats Paw pressure Test 0-240 min of ANA dose 62.5-1,000 mg/kg i.p. data present as mean %MPE ± S.E.M. N=10 per group

Dose ANA (mg/kg)	15 min	30min	60 min	90 min	120 min	240 min
62.5	-0.14±2.06	2.67±2.26	5±3.75	3.55±1.15	2.16±1.77	1.69±2.1
125	4.59±2	5.79±2.25	10.83±6.08	5.26±2.12	4.02±1.42	4.44±1.7
250	12.14±7.9	29.65±1.3	21.71±7.11	3.1±1.7	5.05±1.8	4.73±1.24
500	21.54±8.2	28.62±6.88	13.02±3.74	12.73±4	5.17±1.74	9.62±1.3
750	10.3±2.73	31.68±8.78	31.46±5.35	15.07±3.56	14.3±5.61	22.34±7.74
1000	54.11±1.35	67.54±12.81	52.95±10	46.79±11.31	30.35±8.25	25.09±7.45

Table 4 Effect of ANA on motor impairment in rotarod test. N=6 per group.

Group	Time after ANA injection (Mean ± S.E.M.)			
	30 min	60 min	120 min	240 min
0.9%NSS	180 ± 0	180.0 ± 0	178.4 ± 1.6	180.0 ± 0
ANA 125 mg/kg	168.2 ± 7.27	166.4 ± 9.75	178 ± 2.0	175.8 ± 2.69
ANA 250 mg/kg	166 ± 6.61	164 ± 10.63	177.6 ± 2.4	177.4 ± 2.60
ANA 500 mg/kg	155.4 ± 15.16	150.4 ± 12.3	159.2 ± 13.29	175.2 ± 4.8

VITAE

Miss Janjuree Kaopinpruck was born in December 16, 1970 in Chiang Mai, Thailand. She graduated with a Bachelor of Nurse in 1993 from the Faculty of Nursing, Mahidol University, Bangkok, Thailand. After graduation, she worked in ICU Pediatric surgery, Siriraj Hospital, Bangkok, Thailand.